THE EFFECT OF ULTRAVIOLET AND GAMMA IRRADIATION ON SOLUBLE CALF-SKIN COLLAGEN.

by

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Forword.

This thesis is submitted in accordance with the regulations for the Degree of Doctor of Philosophy of Rhodes University. The work was carried out at the Leather Industries' Research Institute, Grahamstown, and is wholly original except where due reference is made in the text. It has not been submitted in whole, or in part, for any degree at any other University.

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Summary.

Initially the present study was confined to the effects of ultraviolet irradiation on acid-soluble collagen. Such a study was undertaken in order to demonstrate the critical role played by tyrosine and phenylalanine in the intermolecular interaction of the telopeptides protruding from the rigid parent collagen molecule. Since both tyrosine and phenylalanine are photosensitive, and because the collagen telopeptides are relatively rich in these aromatic amino acids, carefully controlled degradation studies involving telopeptide liberation could be made.

It became increasingly apparent during the course of investigation, that a better understanding of the subunit composition of thermally denatured acid-soluble collagen was necessary if a satisfactory interpretation of the irradiation studies was to be made. A subsequent study of the subunit composition of thermally denatured acid-soluble collagen resulted in the isolation and characterisation of two major and two minor dimeric components as well as the α- and β- subunits. Three acidic telopeptides and three basic telopeptides were also isolated from acid-soluble collagen during the course of the present study. The presence of the dimeric components while related directly to the method of preparation, suggested that acid-soluble collagen was largely dimeric in nature.

Such a conclusion suggested an intermolecular rather than an intramolecular relationship between neutral-salt-soluble and acid-soluble collagen.

While it is currently accepted that an intramolecular relationship exists between neutral-salt-soluble and acid-soluble collagen, such a relationship does not satisfactorily explain the very different solubility characteristics displayed by these soluble collagen extracts. With this in mind, and using the study on the subunit composition of thermally denatured acid-soluble collagen as a basis for comparison, the intra and intermolecular relationship between neutral-salt-soluble and acid-soluble collagen was investigated using ultraviolet and gamma irradiation.

The effects of ultraviolet and gamma irradiation on soluble collagen preparations proved very similar. Although collagen samples were irradiated in solution from an ultraviolet source; and under anhydrous conditions from a gamma source, much the same degradation mechanism resulted. The initial depolymerisation of dimeric material followed by peptide fission, yielding irradiation-resistant crystalline portions of the parent triple helix, took place in both instances. At the same time, both studies indicated no significant differences in the intra or intermolecular structures of the neutral-salt-soluble and acid-soluble preparations investigated. The dimer content of neutral-salt-soluble collagen preparations was, however, noted to be smaller.

to current methods for the preparation of soluble collagens, it may be concluded that such preparations are peptideless to some degree. While the native tropocollagen monomer with its full complement of telopeptide side chains may actively undergo linear polymerisation resulting in fibre formation, the soluble collagen preparations referred to above may only aggregate in a rather random fashion.

Contents.

CHAPTER 1.

THE MACROMOLECULAR CHEMISTRY OF COLLAGEN.

Introduction.

The properties of collagen are of interest from many points of view. By virtue of the role played by collagen in the body, a far better understanding of the biosynthesis of collagen is necessary. The treatment of wound tissue as well as the many collagen diseases are of particular interest to the pathologist, while the nature of bone collagen plays an important part in dental and osteological studies. From a commercial point of view, a better understanding of the reaction of collagen with tanning materials has important applied value, whilst chemically modified gelatin together with reconstituted collagen fibre present speculative potential. All these latter aspects are directly related to the complexity of collagen chemistry. It is from this point of view that the biochemist or biophysicist finds collagen of such interest. In this respect, the properties of collagen on the molecular level are unique.

Collagen is the principle protein present in skin, tendon, cartilage, teeth, bone and the white fibrous connective tissue of the body. It comprises 20-25% of the total protein of the animal body (Neuberger and Niebarde, 1964). Although the physiological activity of collagen is relatively slight compared with the globular proteins,

certain enzyme and surface effects play a vital role in body metabolism.

Chemical Composition.

Collagen is unique among the proteins because of its amino acid composition. It is the only mammalian protein containing large amounts of hydroxyproline, while also being exceptionally rich in glycine and proline. Aromatic and sulphur-containing amino acids are characteristically absent. Small amounts of hydroxylysine are also characteristically present. Very close to one-third of the total residues are glycine, about one in ten is hydroxyproline, and twelve in a hundred are proline. Because of the high content of these imino acid residues which have no hydrogen atom on the peptide bonds involving these residues, intramolecularly hydrogen-bond stabilized structures such as the α-helix are not found.

The acidic and basic functional groups of collagen govern many of the physical properties of the collagen fibre, as well as determining the overall reactivity of collagen. In the native state, collagen has a small excess of basic groups and hence should have a basic isoionic pH. The location of the pI for the insoluble collagen fibre is difficult, but a value for the acid-precursor gelatins of 9.0 - 9.2 has been firmly established (Janus et al. 1951). Highberger (1936, 1939) found that the isoelectric point of native collagen was about 7.8. IEP values for acid-soluble collagens and for alkali treated collagen approach a value of 5 due to the binding of small inorganic ions by the collagen (Veis et al. 1958).

The neutral hydroxy amino acids, hydroxyproline, serine, threonine and tyrosine, account for 154 residues per 1000 residues in bovine skin collagen; nearly as many as the total free acidic and basic groups in native collagen. Tyrosine which occurs to only a small extent, about 5 residues per 1000, has been the subject of much conjecture. Eastoe (1955) shows a depletion of tyrosine content of gelatins as compared with the parent collagen and concludes the presence of a tyrosine-rich impurity. Russell (1958) claims to have isolated a tyrosine-rich, hydroxyproline-poor fraction from gelatin which Leach (1960) was not able to confirm. Steven and Tristram (1962) also claim to have isolated a noncollagenous fraction corresponding to about 2% of the total protein nitrogen from acid-soluble collagen. Although the tyrosine content of collagen is low, it would appear to play an important role. Deasy (1959) concluded that some tyrosine was an integral part of the collagen molecule, while Bensusan and Hoyt (1956), and Hodge et al. (1960) have shown that the tyrosine residues play an important part in the aggregation properties of soluble collagen.

Gustavson (1942, 1949, 1950, 1954 and 1956) has discussed the relationship between the hydrothermal stability of collagen and the hydroxyl group content of collagens in great detail. It was shown that collagens from various sources displayed shrinkage temperatures which had a direct relationship to their hydroxyproline content. From this and other supporting evidence it was concluded that an interchain hydrogen

bond existed between the hydroxyl group of the hydroxyproline on the one peptide chain, and the carbonyl group of a peptide linkage on an adjacent chain. These hydrogen bonds are primarily responsible for stabilizing the collagen macromolecule.

Nearly 45% of the residues of collagen are the nonpolar residues of glycine or alanine. This is almost three times the proportion normally found in proteins, yet they contribute little to nonpolar intermolecular van der Waals' interactions or to hydrophobic bonding because of their small size. Ramachandran et al. (1962) point out that the glycine content is crucial in determining the structure of the peptide strands in collagen, and state a definite requirement for a protein to be in the collagen class, is for the glycine content to account for close to one-third of all amino acid residues.

Highly purified collagens also contain small amounts of simple sugars that cannot be removed from the native protein. Hörmann (1960) has shown that the hexose in acid-soluble collagen is glucose, whereas in insoluble collagen both glucose and galactose were found.

The Molecular Organisation of Collagen.

The interpretation of the diffraction pattern of the collagen fibre is generally accepted from the work of Ramachandran et al. (1954, 1955 and 1956) which was later verified by Cowan et al. (1955 a and b), Bear (1956) and Rich and Crick (1955, 1958 and 1961). The molecular structure consists of three separate peptide chains, coiled along a

left handed threefold screw axis. Each residue takes one - 120° turn around the axis, or three residues complete one turn. The pitch of each coil is such that the three residues complete their turn in 9A. The three chains are bonded together with their axes parallel and aligned so that a translation perpendicular to the axis of any one chain takes one to a similar site on either of the neighbouring chains. Every third peptide group along each chain will be in an identical environment, either near the middle of the group of three chains or near the outside. When the chains are properly oriented, the third NH group on the backbone of one chain can make a hydrogen bond with every third carbonyl group on the backbone of a neighbouring chain. Rich and Crick (1961) point out that two such structures are possible in which the chains are "phased" with respect to each other. Structure II may be created from structure I merely by rotating each individual chain about its own axis by about 60°.

The Structure of the Collagen Fibril.

Turning now to the large-scale features of the collagen complex, such techniques as wide-angle and small-angle X-ray diffraction, as well as electron microscopy have proved most valuable. Viewed microscopically, collagen appears to be laid down in bundles made up of parallel fibrous elements characterized by a distinctive pattern of fine cross-striations. At high resolution in suitably stained preparations, these striations are seen to consist of regularly repeating sets of "bands" and "inter-

bands" having fixed relative positions and electron densities. The over-all pattern repeats at an average axial interval of about 640A (Schmitt and Gross, 1948).

Since a fundamental axial repeat could be observed by means of both electron microscopy and X-ray diffraction, it appeared that this spacing represented a specific repeating structural unit. Bear (1952) suggested that the 640A band - interband repeat was due to the alternation of groups of amino acids, the band region being rich in long-chain polar residues, and the interbands containing mostly smaller non-polar residues. Thus the bands would be the most amorphous regions of the fibril, and the interbands more crystalline.

This 640A native type periodicity was only later explained using reconstituted collagen preparations, the collagen being precipitated in the "fibrous long spacing" form (Gross et al. 1952 ; Randall et al. 1952) or the "segment long spacing" form (Schmitt et al. 1955 ; Schmitt, 1956). These forms have been shown to be completely interconvertable.

Schmitt and co-workers have subsequently interpreted these results in terms of a polarized collagen molecule, "tropocollagen", which they deduced should be about 2600A in length and 15 - 20A in diameter (Fig. 1).

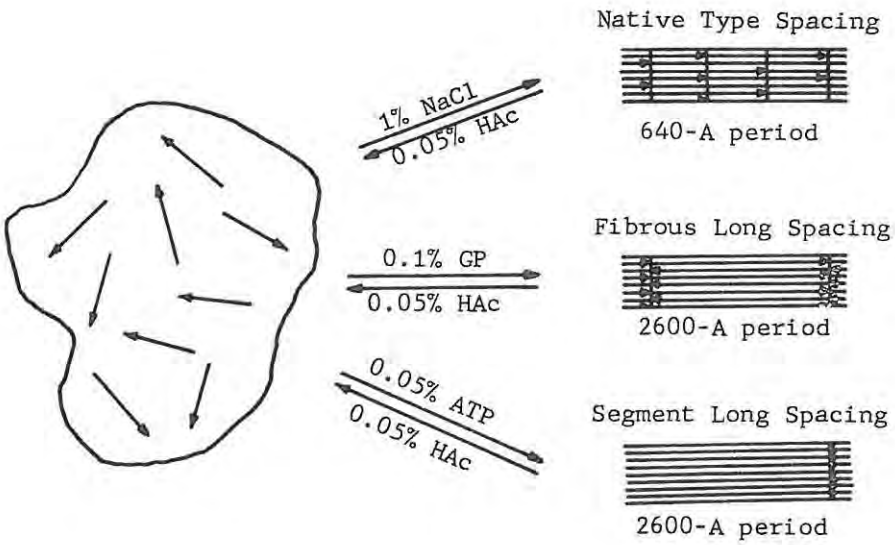

FIG. 1. The Macromolecular Organisation of Tropocollagen Monomer Units.

concluded that the mode of end-to-end polymerization of TC monomers

Soluble Collagen : Tropocollagen.

Zachariades (1900) was the first to observe that some collagen would dissolve in cold dilute acetic acid. Much later Orekhovitch et al. (1948) demonstrated that soluble collagen could be obtained from any collagen, and in particularly large amounts from the tissues of young animals. Basically, the procedure involved the extraction of minced connective tissue with dilute citrate buffer at pH 3.5 to 4.0.

Over the last few years it has been shown by several groups that collagen may also be dissolved in a variety of neutral salt solutions (Gross et al. 1955 a ; Jackson and Fessler, 1955 ; Gallop et al. 1957 a), and in mild alkali (Harkness et al. 1954).

The collagen molecules extracted by these various procedures are essentially identical in terms of size, shape, chain configuration, and most physicochemical properties. The monomer unit was named tropocollagen (TC) (collagen-former).

The physicochemical measurements made by Boedtker and Doty (1956) indicated that the TC unit was composed of three peptide chains with an overall molecular weight of about 340,000. The dimensions of the ordered part of the three chain unit matched those predicted by the electron microscope and X-ray diffraction evidence. However, the weights and hence the chain lengths were not found to be equal, suggesting the possibility that the three-stranded TC unit had freely

dangling side chains at one or both ends. Hodge and Schmitt (1958) concluded that the mode of end-to-end polymerization of TC monomers involved the coiling of these free terminal chains about each other to form highly ordered, possibly helical structures. These suggestions must be modified in view of the work by Petruska and Hodge (1963) but, as shown by Gallop (1964), the end regions of the TC monomer is of specific importance in controlling the mode of aggregation of the TC units, and in modifying the properties of the intact fibre.

The arrangement of TC units in the native fibre was deduced by Hodge and Schmitt (1960) to be that of a staggered array in which adjacent TC units were shifted, in any particular plane, through a fibril perpendicular to the fibre axis, by one quarter of their length. The TC units are thought to be 3000A in length and a diameter of 13.6A, with a staggered end-region overlap of $\sim$ 280A causing the characteristic $\sim$ 700A spacing to appear (Fig. 1). These dimensions were confirmed by Rice (1960) by direct electron microscope observation of single collagen molecules.

The Subunit Composition of Soluble Collagen.

The conversion of collagen to gelatin can normally be accomplished only by severe chemical treatment. Soluble collagen, however, can be converted to gelatin by gentle warming on by the use of lyotropic agents. It has been found that several discrete components are produced by such treatments. Gallop (1955) called the gelatin

produced in this fashion "parent gelatin", and concluded that parent gelatin should be the highest molecular weight gelatin obtainable. The subsequent inter-relationship of these sub-units to each other and to the collagen macromolecule provides powerful evidence for the intramolecular cross-linking in collagen. The first separation of soluble collagen into two components is attributed to Orekhovitch and Shpikiter (1955) who postulated that procollagen consisted of two or more components held together by weak bonds that were broken irreversibly on thermal denaturation. The components were identified by ultracentrifugation of denatured citrate-soluble collagen solutions at very low concentration. The smaller slower-sedimenting component was designated the α -component, and the larger faster-sedimenting component termed the β -component (Orekhovitch and Shpikiter, 1957). In addition to the α- and β -components, a heavier, more rapidly sedimenting component generally known as the γ-component was first reported by Veis et al. (1960).

Where other methods are used for subunit separation, the components obtained have normally been characterised by ultracentrifugation. The uses and limitations of such characterisation, have however, been critically reviewed by Hannig and Engel (1961). Aggregation of the subunits must be prevented either by sedimentation at temperatures in excess of the denaturation temperature or by sedimentation in the presence of a lyotropic reagent.

Ultracentrifugal separation, although the preferred method for component characterisation, is not applicable to their preparative isolation. The first attempts to obtain the α- and β- components on a preparative scale were made by Orekhovitch and Shpikiter (1957) using ammonium sulphate fractionation. Only partial enrichment of either component was thus achieved. Chun and Doty (1958) using a similar method of alcohol coacervation eventually prepared a homogeneous α-component. Veis et al. (1960) similarly prepared a homogeneous α-component from gelatin extracted from collagen at 60°.

The separation of degraded soluble collagen on carboxymethylcellulose columns has proved a valuable tool in recent years. Kessler et al. (1959, 1960) obtained between four and six peaks depending on the concentration of acetic acid used to dissolve the collagen. The earlier peaks were low molecular weight fragments, but the last three peaks were non-dialysable. Unfortunately these latter fractions were not related to the known α- and β- components.

Piez et al. (1960) fractionated denatured acid-soluble collagen on CM-cellulose and obtained two main fractions each of which was contaminated by some further material. The major components of the fractions, however, appeared to correspond to the α- and β- components when subjected to ultracentrifugation. In later work (1961, 1963) four components were separated, two of which corresponded to α-components, and two to β- components. Both Highberger (1961) and Schleyer (1962) have also reported on the use of CM-cellulose for the

separation of α - and β - components. Schleyer found two peaks in addition to those of Piez et al.

Electrophoretic studies of soluble collagens and their denaturation products have been carried out (Tomlin and Turner, 1957 ; Astrup et al. 1958 ; Young and Lorimer, 1960), but the results have only recently been related to the α - and β - components (Nänto et al. 1963, 1965 ; Hollmèn and Kulonen, 1964 a and b ; Nagai et al. 1964 ; Reich, 1964). In several instances the presence of additional uncharacterised "X" components were also reported (Hollmèn and Kulonen, 1964 b ; Nagai et al. 1964 ; Nänto et al. 1965).

Before considering certain features of these components and their relationship to each other and to the TC molecule, it is important to emphasize that the α - and β - and probably the γ - component can be prepared from acid-soluble collagens obtained from a variety of animal and fish tissues. It is evident that at least two discrete components result from the thermal denaturation of acid-soluble collagen irrespective of the source of the parent material.

A summary of the molecular weights of the α - component ($M\alpha$) and the β - component ($M\beta$) made by Hannig and Engel (1961) and Harding (1964), indicate $M\alpha \sim 100{,}000$ and $M\beta \sim 200{,}000$ while $M\gamma \sim 300{,}000$. This would suggest that the β - component is made up of two covalently bound α - components, and that the γ component consists of three covalently bound α - components constituting the intact TC molecule.

Altgelt et al. (1961) have clearly demonstrated the ability of the γ - component to renature to the SIS form of collagen.

In view of the apparent interconversion of these components it might be expected that an integral proportionality does not always exist between the α - and β - components. Ratios ranging from 5 : 2 to 1 : 1 for denatured acid-soluble collagens are reported (Harding, 1964). In many instances, however, salt-soluble collagen has not been extracted first, and the reported ratios therefore relate to a mixture of salt- and acid-soluble collagen. It has been shown that both salt-soluble (Mazurov and Orekhovitch, 1960 ; Orekhovitch et al. 1960 ; Piez et al. 1961, 1963 ; Nänto et al. 1963) and alkali-soluble (Mazurov and Orekhovitch, 1960 ; Nikkari and Kulonen, 1962) collagens consist almost entirely of α - components. As expected, a lower $\alpha : \beta$ ratio is found when the salt-soluble collagen is first removed.

The first amino acid estimations carried out on the α - and β - components were reported by Orekhovitch and Shpikiter (1957) ; Chun and Doty (1958) and later by Mazurov and Orekhovitch (1960) and Piez et al. (1960). It was only later shown by Piez et al. (1961) that the so-called α - and β - components were in fact binary mixtures (α_1 and α_2 ; β_{11} and β_{12}). They succeeded in isolating and analysing all four subunits, and so demonstrated definite differences in composition between them. Clearly the α - and β - components studied hitherto were mixtures. The authors found that while the compositions of α_1 and α_2 differed, that of β_{11} was similar to that of α_1, and that of β_{12} was the mean

composition of α_1 and α_2. All the components had a composition typical of collagen. On this basis and on the molecular weight relationship they suggested that β_{11} consisted of two α_1 chains linked together, while β_{12} consisted of an α_1- and an α_2-chain joined together. Both the results of Schleyer (1962) and subsequent reports by Piez et al. (1963) have added support to these findings.

Earlier observations by Chun and Doty (1958), Doty and Nishihara (1958) and Astrup et al. (1958) had indicated that the action of both heat and alkali treatments increased the relative proportions of the α - component. It therefore appeared that the β - component consisted of two α - components held together by an alkali-sensitive bond. This supposition has, however, not been entirely accepted by more recent findings. Young and Lorimer (1960) have observed intact β - components in denatured cod-skin that had previously been subjected to alkaline treatment at pH 13. Altgelt et al. (1961) found a more general molecular breakdown of α -, β - and γ - components by heating to 70°, while Mazurov and Orekhovitch, (1960) found no degradation of the β - subunit at pH 9.

More recently Schleyer (1962) investigated the effect of alkali on the parent TC solutions as well as the individual components. The author found that although the β - components were degraded, the products were not α - components although they displayed the chromatographic character of α_1 components when separated on CM-cellulose. A molecular weight for the degraded β - components of about 50,000 was reported.

Kuhn et al. (1963) recently found that both soluble and insoluble collagen were converted to a single α- component by the action of alkali. It would appear at this stage that the possible $\beta \rightarrow \alpha$ conversion is not as yet satisfactorily explained.

Further conflicting evidence concerning the denaturation of soluble collagens has been reported by Bakerman and Hersh (1964). Soluble collagen extracted from human skin was found to contain only β - components. This was interpreted by the authors as suggesting that the TC collagen monomer "must be a whole-number multiple of the molecular weight of the β -chain".

Piez (1964) has since demonstrated the non-identity of the three α -chains in codfish skin collagen, while Bornstein et al. (1964) have identified a new β - component viz. β_{22} constituting of two α_2- components linked together.

Unknown Components.

Schleyer (1962) using a similar technique to that of Piez et al. (1960) first reported the presence of a large γ - component in addition to the α - and β - components. This component was eluted with a 0.5M acetate buffer (pH 4.8) following the gradient elution of the α - and β - components. The γ - component was not characterised.

At about the same time Kulonen et al. (1962) using separation on ion exchange resins isolated a "sodium hydroxide fraction" which was

thought to resemble the parent collagen by virtue of a similar pI.

Using electrophoretic techniques both Hollmèn and Kulonen (1964) and Nagai et al. (1964) reported the presence of additional X-components as being "large aggregates" of denatured collagen. Nänto et al. (1965) using similar techniques reports the presence of two such X-components.

Tristram et al. (1965) separated out "components 3 and 4" using CM-cellulose according to the method of Piez et al. (1963). Component 3 was related to the γ -component of Schleyer and thought to be a polymerised form of α -chains held together by hydrogen bonding. Component 4 was thought to resemble the parent collagen and was related to the "sodium hydroxide fraction" obtained by Kulonen et al. (1962); which would correlate with the γ -component reported by Altgelt et al. (1961) and Grassmann et al. (1961).

Cooper and Davidson (1965) also report the presence of a large X-component using a modification to the method of Piez et al. (1963). The component was not characterised although a molecular weight equal to or less than the β - subunit was indicated.

Although the presence of these unknown components together with the conflicting evidence presented in the previous section does not correlate with the current hypothesis as suggested by Piez et al. (1963), the latter hypothesis is currently supported by the majority of workers in this field (see Summary by Harding, 1964). The intramolecular relationship of the subunits to the parent tropocollagen molecule is

diagramatically presented in Fig. 2.

While the currently accepted intramolecular relationship between neutral-salt-soluble and acid-soluble collagen has certainly laid a foundation upon which further development may take place, it does not satisfactorily explain the marked differences in solubility displayed by these soluble collagens. It was from this point of view that the present study was undertaken.

In order to illustrate an intramolecular relationship between neutral-salt-soluble and acid-soluble collagen, a degradation study of the individual preparations under similar conditions should reveal significant differences. In the present thesis the degradation of neutral-salt-soluble and acid-soluble collagen using ultraviolet and gamma irradiation has been investigated. These studies revealed no significant differences between neutral-salt-soluble collagen and acid-soluble collagen as currently prepared by workers in this field.

Initial investigations, however, into the thermal denaturation of acid-soluble collagen resulted in the isolation of four unknown major components. Evidence for the dimeric nature of these components, as well as the dimeric nature of acid-soluble collagen is discussed. Furthermore, evidence for the monomeric nature of neutral-salt-soluble collagen strongly suggests that an intermolecular rather than intramolecular relationship between neutral-salt-soluble collagen and acid-

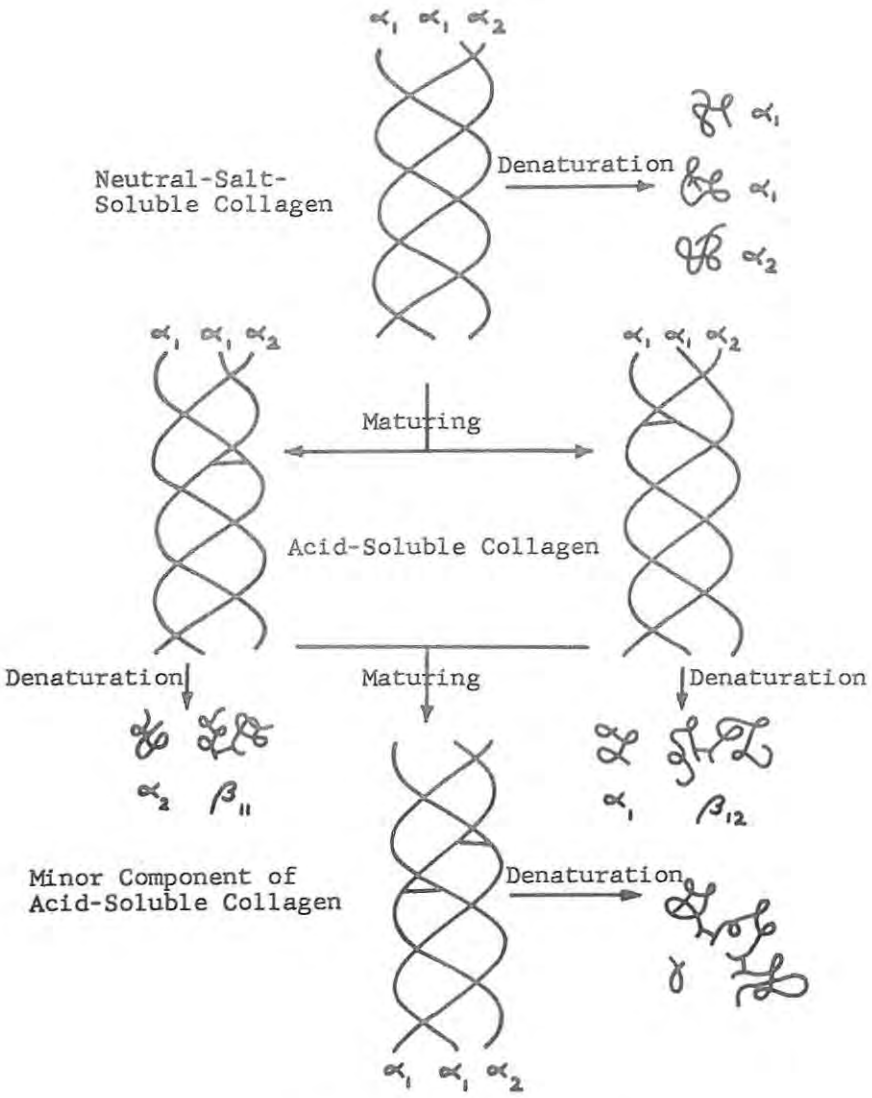

FIG. 2. The Biosynthetic Introduction of Intramolecular Cross-links into Collagen.

soluble collagen exists. Evidence for the biosynthesis of collagen by means of a linear polymerisation mechanism involving highly active monomeric units is presented.

CHAPTER II.

MATERIALS AND METHODS.

PREPARATION OF SOLUBLE COLLAGEN.

At present the physicochemical properties of the tropocollagen molecule are not clearly defined. Variants such as the tissue source and the method of preparation exert considerable influence on such properties, and must be taken into account. Though differences in chemical composition from one variant to another may be small, the differences in biochemical and physiological reactivity may be great. For such reasons, no standard methods for the preparation of soluble collagens are currently in use, while standards of purity of such preparations are not strictly defined. Many methods for the extraction of acid-soluble collagen appear in the literature, all of which rely on an initial extraction using either dilute acetic acid or citrate buffers. In many instances the removal of neutral-salt-soluble collagen prior to the acid extraction is not carried out, resulting in considerable confusion in some cases.

It became increasingly apparent during the course of the present study that the significant physicochemical differences displayed by soluble collagen preparations may be related to a large extent to the method of preparation (see Chapter VI). Preliminary investigations confined to the subunit composition of acid-soluble collagen related the presence of dimeric components directly to the

method of preparation. At the same time, these findings suggested an intermolecular rather than intramolecular relationship between acid-soluble and neutral-salt-soluble collagen. The initial purpose of this investigation was to study the effects of ultraviolet and gamma irradiation on acid-soluble collagen, but for reasons outlined briefly above and discussed in detail in Chapter VI, it was also considered necessary to study the relationship between neutral-salt-soluble and acid-soluble collagen. Consequently preparations of both neutral-salt-soluble and acid-soluble collagen were made. Six individual calf-skin extracts from different breeds of animal and varying in age from one week premature to four weeks old were prepared. All extraction operations were conducted in a cold room ($0-4^{\circ}$), while centrifugation was carried out in an MSE preparative centrifuge at 5° using either a normal or a continuous action rotor. The extraction procedures are described below.

Neutral-Salt-Soluble Collagen.

Preparation No. 1 : Soluble collagen was extracted using the method of Gross (1958) as modified by Piez et al. (1963). The skin of a freshly slaughtered four-week-old Jersey bull-calf was defleshed and macerated with ice through an electrical mincing machine. The ground tissue was extracted with about ten volumes of 10% NaCl with intermittent stirring for 48 hours. The extract liquor was filtered through cheese cloth and then through cotton wool. This extraction

was repeated three times, and the combined liquors filtered under vacuum through Whatman No. 541 filter paper. The filtrate was then acidified with acetic acid to pH 3-4, and the salt concentration diluted to 5%. It was found that at the 10% salt concentration no settling out of the collagen precipitate took place, but that on diluting to 5%, the precipitate readily floated to the surface thus facilitating the partial separation of the bulky subnatent liquor from the precipitate. The combined precipitates were collected by centrifugation at 6,000 g for 30 min. This precipitate was then extracted with $1\frac{1}{2}$ litres 0.5 M-HAc with continuous stirring for 48 hours and the soluble collagen clarified by centrifugation at 30,000 g for $1\frac{1}{2}$ hours. It was noted that even after this exhaustive extraction with acetic acid, a large insoluble fraction of the original precipitate remained. The acetic acid extract was then made up to 5% ($^{w}/_{v}$) NaCl and the collagen allowed to precipitate. This was collected by centrifugation at 6,000 g for 60 min, taken up in 0.5 M-HAc and then centrifuged at 30,000 g for 90 min. The collagen precipitate now appeared to be readily soluble in dilute acetic acid at this stage. This solution was then dialyzed against 0.02 M-Na_2HPO_4 for 4-5 days with two changes of buffer, and the precipitated collagen centrifuged off at 6,000 g. The precipitate was then taken up in 0.15 M-HAc and dialyzed against 0.15 M-HAc for 24 hours before centrifuging at 30,000 g for 2 hours. This preparation was then lyophilized and stored over silica-gel at 0-4°.

Preparation No. 2 : Soluble collagen was extracted from the skin of a freshly slaughtered Friesland heifer-calf, adjudged to be about four weeks premature, using the same method as in preparation No. 1. It was again noted that the initial collagen precipitate from the salt extraction was largely insoluble in 0.5 M-HAc. This observation is discussed in Chapter VI.

Preparation No. 3 : A new method for the extraction of neutral-salt-soluble collagen was used.

The skin of a freshly slaughtered 5 day old Jersey heifer-calf was macerated and extracted with 10% NaCl as in preparation No. 1. This extraction was limited to 12 hr. After filtering through Whatman No. 541 filter paper the extract was centrifuged at 35,000 g for 60 min. The supernatent extract was then dialyzed against distilled water overnight (pH 7.1), and the precipitate collected at 35,000 g for 60 min. This precipitate was then taken up immediately in IN-NaCl and dialyzed against distilled water for three days with three changes of water. The resulting precipitate was collected by centrifugation at 35,000 g for 60 min and then freeze dried and stored over phosphorus pentoxide at 0-4°.

It was noted that in all three neutral-salt-soluble collagen preparations, considerable discoloration of the initial collagen precipitate resulted, and that the extent of discoloration was dependant on the time taken in extracting the macerated tissue with 10% NaCl.

These observations suggest that a very short extraction procedure is necessary in preparing neutral-salt-soluble collagen. Acid-soluble collagen preparations did not display such an affinity for occluded impurities. This observation is also discussed in Chapter VI.

Acid-Soluble Collagen.

Preparation No. 1 : Acid-soluble collagen was extracted using the method of Gross (1958) as modified by Piez et al. (1963).

Following the extraction of neutral-salt-soluble collagen as detailed under preparation No. 1, acid-soluble collagen was extracted from the residual tissue with about 10 volumes of 0.5 M-HAc for from 3-5 days. The suspension was filtered through cheese cloth and then twice through Whatman No. 541. The extraction was repeated resulting in a total of 12 litres of solution. Acid-soluble collagen was then precipitated by the addition of 5% NaCl ($^{w}/v$) and the precipitate collected by centrifugation at 6,000 g for 45 min. The precipitate was taken up in 0.5 M-HAc and centrifuged at 30,000 g for 60 min. The collagen was then dialyzed against 0.02 M-Na_2HPO_4 and purified as described for neutral-salt-soluble collagen.

Preparation No. 2 : Following the extraction of neutral-salt-soluble collagen as detailed under preparation No. 2, acid-soluble collagen was prepared from the residual tissue using the same method as in preparation No. 1.

action rotor at 18,000 g, and the collagen precipitated with 5% (w/v)

Preparation No. 3 : Acid-soluble collagen was extracted using the modified method of Steven and Tristram (1962).

The skin of a freshly slaughtered bull calf ajudged to be about one week premature was immediately defleshed, washed and stored in ice at -12° for 3 days prior to extraction. The animal was an Afrikander-Shorthorn crossbred type.

The whole skin was macerated together with liberal quantities of ice through an electrical mincing machine, and the macerated tissue (4 Kg.) suspended in 10 l of 0.2 M-NaCl in order to remove neutral-salt-soluble collagen. This procedure was repeated four times for periods of 36, 24, 24 and 48 hours respectively while maintaining intermittent agitation. The pH measured after each extraction averaged 7.5.

Acid-soluble collagen was extracted from the residual tissue with 10 l of 0.5 M-HAc. The pH of the mix was adjusted to between 3.8 and 4.0 using HAc, and the extraction continued for 24 hours with occasional stirring. The extraction was repeated three times using 0.01 M-HAc in the subsequent extractions. The use of 0.5 M-HAc was not found necessary in maintaining the pH at 3.8 - 4.0 in subsequent extractions due to the buffering capacity of the already acidified macerated tissue.

The combined extracts were then clarified through a continuous action rotor at 18,000 g, and the collagen precipitated with 5% (w/v) NaCl. The precipitate was collected by centrifugation at 18,000 g using a continuous action rotor, taken up in 0.01 N - HAc, and again centrifuged at 18,000 g. This precipitation procedure was then repeated and the precipitated collagen lyophilized and stored over silica-gel at 0-4°.

Because the individual yields of neutral-salt-soluble and acid-soluble collagen were not of interest in the present study, no attempt was made to record such values.

PHYSICOCHEMICAL METHODS.

Because of the physicochemical nature of the present study, a wide range of analytical techniques was employed. Because these techniques were only used for analytical purposes and not as aspects of investigation, only a brief description of the individual instrumental methods is presented. Where necessary, further detail concerning the experimental procedures used is presented in the relevant chapters.

Viscometry.

Viscosity measurements were made in a Cannon-Fenske viscometer (size 50, B.S. 188) and viscosities calculated according to the certified constants supplied by the manufacturer as follows:

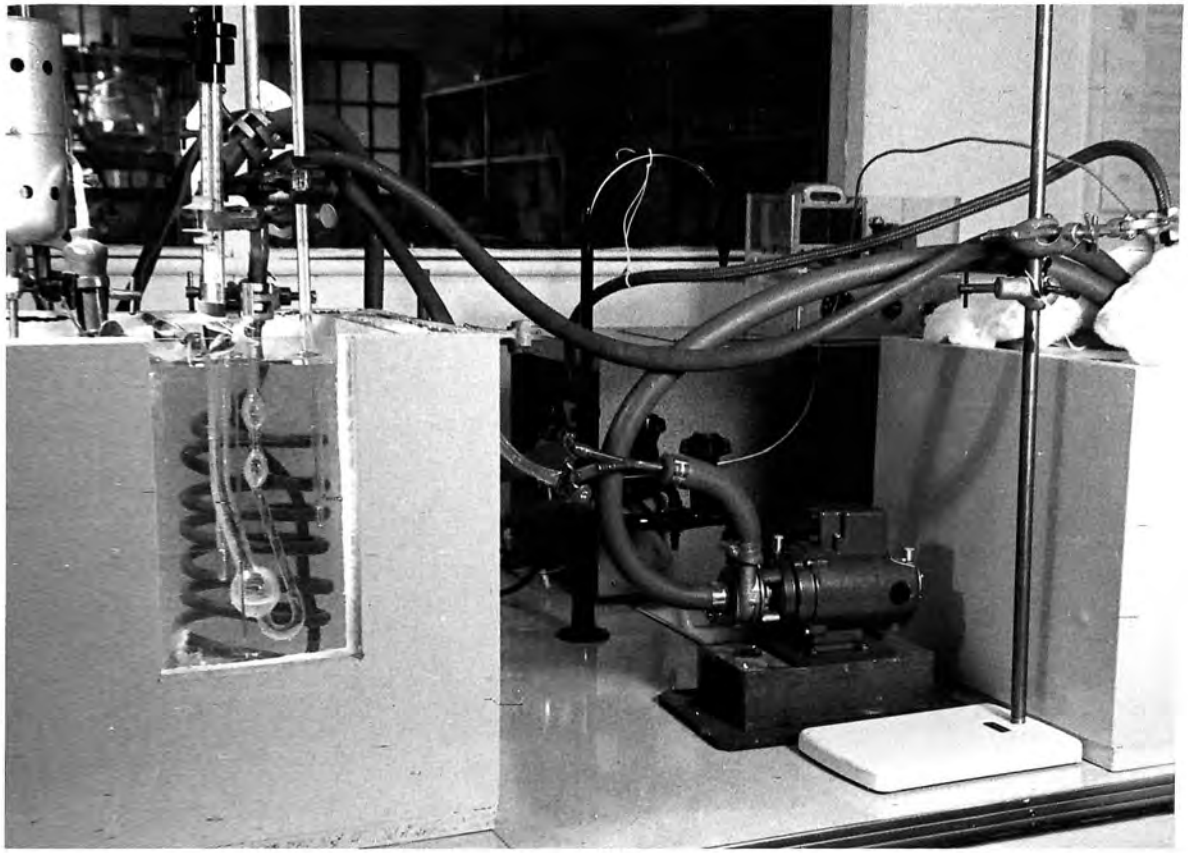

Plate 1. Viscosity Measurements Carried Out Under Controlled Temperature Conditions.

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

where V is the Kinematic viscosity in centistokes, t is the observed flow time in seconds, $C = 0.00338^{7}$ centistokes per second at 100°F, and B = -2.5 centistokes seconds.

The viscometer was cleaned before each measurement with 50/50 nitric acid as prescribed by Janus and Darlow (1962) for gelatin solutions, and then rinsed with distilled water and dried with acetone. The acetone (GR grade) used for drying was initially clarified by passing it through a No. 2 grade sintered filter-disc.

Protein solutions were clarified by centrifuging at 32,000 g and 5° for 60-90 min before pipetting in a 5 ml aliquot for viscosity measurement. In the case of kinetic studies and irradiation studies, protein solutions were centrifuged as described, before the commencement of the prescribed studies.

Viscosity measurements were made on solutions containing 0.1 - 1.4 mg of protein per ml of buffer at temperatures controlled to ± 0.02°. Operating temperatures ranged from 10-45°. At temperatures below 20°, the cooling spiral in the Gallenkamp viscometer bath was coupled to a Colora waterbath maintained at a lower temperature by means of a Colora model TK 64 cold-finger (Plate I).

Plate 11. The Perkin - Elmer Model 141 Polarimeter.

Because a suitable range of accurately calibrated viscometers was not available, the measurement of intrinsic viscosities of collagen preparations at low rates of shear were not possible.

Polarimetry.

Optical rotation measurements were made either in the 40 cm tube of a Bellingham and Stanley polarimeter equipped with a sodium lamp, or in the 10 cm tube of a Perkin-Elmer model 141 digital read-out polarimeter equipped with sodium and mercury lamps (Plate II). Readings were recorded at 589, 578, 546, 436 and 365 mμ respectively using the latter instrument. Measurements were made on solutions containing 0.4 - 1.4 mg of protein / ml of buffer at temperatures controlled to $\pm$ 0.02° by means of water-jacketed tubes. Operating temperatures were maintained at either 15° or 20° using a Colora waterbath and Colora model TK 64 cold-finger.

Chromatography.

Because the method of Piez et al. (1963) for the fractionation of thermally denatured soluble collagen on CM-cellulose was found to give insufficient resolution of the subunits, components and telo-peptide residues found present in both neutral-salt-soluble and acid-soluble collagen (Chapters III and V), new methods were developed. Experimental variants such as column dimentions, grades of cellulose,

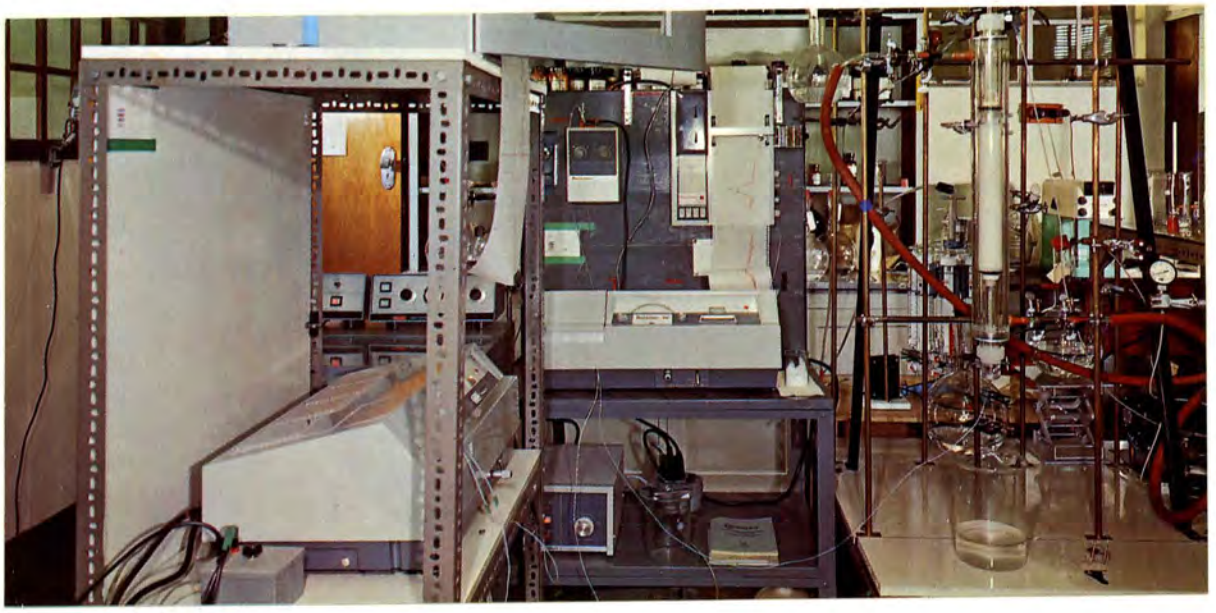

Plate 111. Column Chromatography On CM- Cellulose Carried Out At Two Wavelengths using Two Beckman Model DB Spectrophotometers.

buffer gradients as well as buffer changes were investigated using two different instrumental monitoring systems. These instrumental systems are briefly described below:

(a) Denatured collagen preparations were fractionated on columns of CM-cellulose at 40°. The column effluent was monitored at 230 mμ using a 1 cm continuous flow-cell and a Beckman model DB spectrophotometer and recorder. Eluting buffers were pumped through the system using a Beckman Accu-Flo pump. Buffer gradients were obtained using either a simple two chamber device (Piez et al. 1963), or a nine chamber Buchler Varigrad gradient system. Column elution using buffer changes of increasing ionic strength was also used. Effluent fractions were collected in a Beckman refrigerated fraction collector maintained at 5°. (Plate III). Further experimental detail is presented in Chapter III.

(b) Denatured collagen preparations were similarly fractionated on CM-cellulose at 40° using the Beckman model 130 Spectrochrom Analyzer (Plate IV). Effluent absorption was continuously recorded at 220, 230 and 278 mμ respectively at two optical path lengths (2.5 mm. and 10 mm.) so that a wide range of concentrations could be analyzed. Both pH and conductivity were also recorded. Further experimental detail is presented in Chapter V.

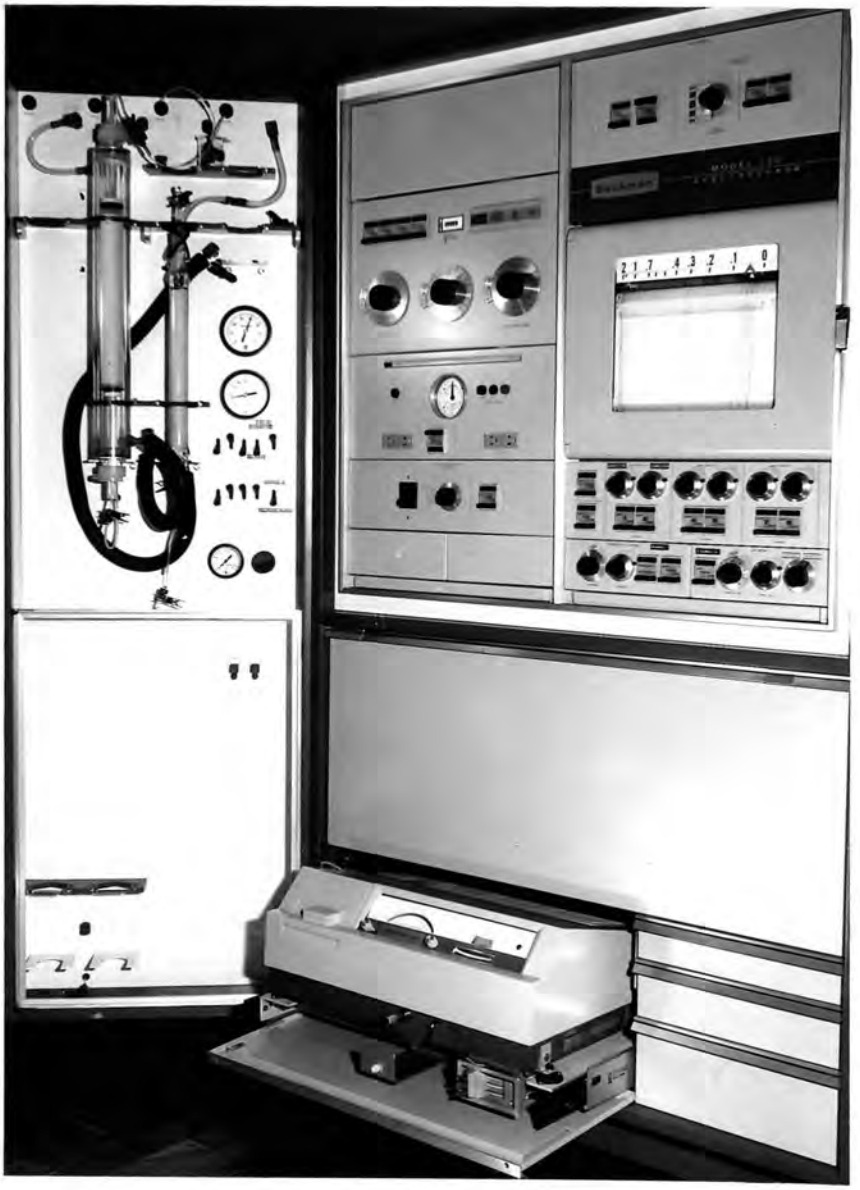

Plate 1V. The Beckman 130 Spectrochrom Analyser.

Gel-Filtration.

Collagen preparations were fractionated on columns of Sephadex G100 and G200 at 20 or 30°. The column effluent was monitored at 230 mμ using a 1 cm continuous flow-cell and Beckman model DB spectrophotometer and Philips recorder. Eluting buffers were pumped through the system using an Accu-Flo pump and effluent fractions collected in an LKB fraction collector maintained at 15°.

Amino Acid Analysis.

The amino acid compositions of freeze dried protein preparations were determined using either the Beckman 120B Analyzer or the Beckman Unichrom Analyzer. A typical program used on the Unichrom Analyzer (Plate V) is outlined below:

The column for eluting the acidic and neutral amino acids (55 cm x 0.9 cm) was operated at 33 ml/hr, pumping 0.20 N-citrate buffer (pH 3.25) for 5.9 hr at 30°, changing to 0.20 N-citrate buffer (pH 4.25) at 55° for a further 3.0 hr. The column for eluting the basic amino acids (22 cm x 0.9 cm) was operated at 50 ml/hr, pumping 0.38 N-citrate buffer (pH 4.26) at 33° for 2.09 hr, changing to 0.35 N-citrate buffer (pH 5.28) at 55° for a further 2.09 hr. The ninhydrin reagent (2% $^{w}/_{v}$ ninhydrin, 0.8% $^{w}/_{v}$ stannous chloride made up in 75 parts methyl cellusolve and 25 parts 4 M-NaAc) was pumped into the reaction coil at 100° at 16 ml/hr (acidics and neutral column) and 25 ml/hr (basics

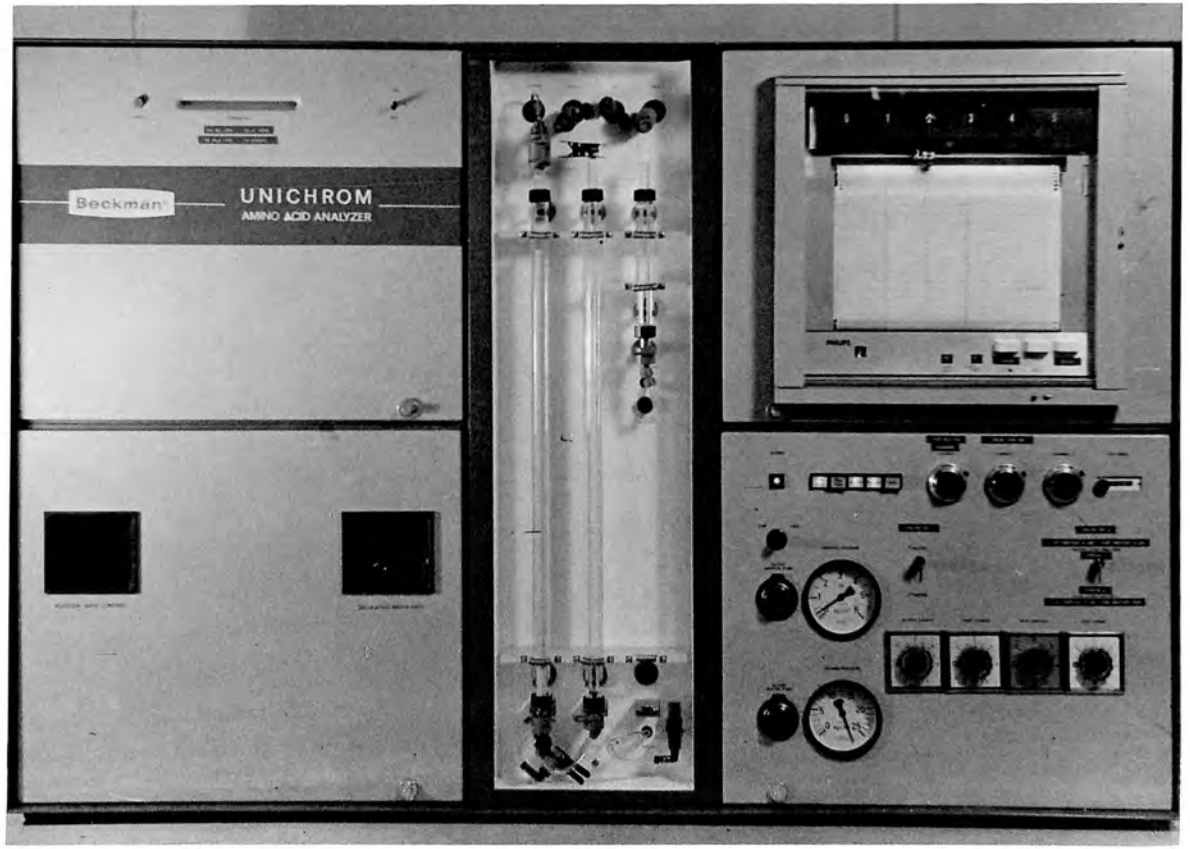

Plate V. The Beckman Unichrom Analyser

column) respectively. Continuous photometric measurements were recorded at 570 mμ (at two path lengths), and 440 mμ (one path length). All the analyses in the present study were carried out using a spherical type sulphonated styrene copolymer resin (7.5% cross-linked).

Protein samples, weighed out on a micro or semi-micro balance, were hydrolyzed with 6.3 N-HCl (2 ml/mg of dry protein) under vacuum at 100° for 24 hr. After rotary evaporation to dryness at 40°, samples were taken up in 0.06 N-citrate buffer (pH 2.2) adding 2.0 ml/mg of dry protein. Analyses were carried out on 1.0 ml hydrolyzate, adding 0.5 μ mole norleucine as a check on the ninhydrin colour development.

High Voltage Electrophoresis and Paper Chromatography.

The amino acid composition of the telopeptides isolated by column chromatography (Chapter V) were studied using high voltage electrophoresis and paper chromatography. The two-dimentional separation of amino acids was achieved using the method of Efron (1959) as modified by Rey et al. (1962). One-dimentional high voltage electrophoresis was carried out on the Shandon H.V.E. at 6,000 V for 25 min at a plate pressure of 1 Kg/cm^2, in a buffer medium consisting of 100 ml formic acid (85-90%) and 150 ml acetic acid made up to 2.5 l (pH 1.6). Chromatography was then performed for 24 hr by a descending technique in a direction perpendicular to that of electrophoresis, with a mixture of 2, 6-lutidine, collidine and water in the proportion 1 : 1 : 1.

After evaporation of the solvent, the spots were located by spraying with a solution of 0.2% ninhydrin in acetone plus 3% ($^{v}/v$) acetic acid. They were then fixed with a solution of copper nitrate.

Isolated collagen components were hydrolyzed and taken up in citrate buffer (pH 2.2) as described under amino acid analysis. These results were compared with the separation achieved using a standard mixture of amino acids.

Ultracentrifugation.

Denatured collagen and collagen fragments were examined by sedimentation velocity at 35° by employing schlieren optics in a Spinco model E ultracentrifuge. The use of such a high operating temperature (35°) also required recalibrating the rotor at this temperature.

Weighing of Sample Preparations.

For subsequent physicochemical analyses, freeze-dried collagen preparations were weighed out as follows:

An approximate weight of material was transferred to a small aluminium-foil weighing boat which was then dried under vacuum over phosphorus pentoxide for at least 12 hr. After attaching a train of U-tubes containing silica-gel, dry air was slowly introduced into the sample desiccator. Weighing was then carried out repeatedly at $\frac{1}{2}$ min

intervals and the dry weight of protein calculated by extrapolation to zero time.

Besides the facility of operation and the saving of preparations, the method compared very favourably with that of oven drying at 105° using an equilibrium moisture content.

CHAPTER III.

THE SUBUNIT COMPOSITION OF ACID-SOLUBLE CALF-SKIN COLLAGEN.

Introduction.

The thermal denaturation of acid-soluble collagen at 40° has been shown to disrupt the tropocollagen helix yielding the subunits α_1, α_2, β_{11}, β_{12} and γ (Piez et al. 1961, 1963). These authors have also demonstrated that the primary structure of the triple helix may be represented by the formula α_1, α_1, α_2 where the α_1- and α_2- chains have molecular weights of about 100,000, but different amino acid compositions. Depending on the age of the collagen, two β - subunits designated β_{11} and β_{12} were also reported, each comprising two covalently bound α - chains. Small proportions of the γ - subunit, comprising three cross-linked α - chains were also isolated.

A third major component of heat denatured acid-soluble collagen designated as component Y, was reported by Schleyer (1962) using chromatographic techniques similar to Piez et al. (1963), while Tristram et al. (1965) have likewise isolated two additional major components, designated components 3 and 4 respectively. Hollmèn and Kulonen (1964) also report the presence of a large X- component using starch-gel electrophoresis, while Francois and Glimcher (1965) using gel-filtration and gel-electrophoresis report the presence of three

components designated x, y and z. The present investigation has been directed at determining the properties and relation to the collagen molecule of four such additional components prepared by the thermal denaturation of acid-soluble calf-skin collagen using a modified chromatography procedure.

In order to avoid confusion in the present study, the term component is used to describe unidentified collagen fractions, while the term subunit refers to the already characterized α, β and γ fractions of thermally denatured soluble collagen.

Materials and Methods.

Acid-Soluble Collagen.

Preparation No. 1. Acid-soluble collagen was prepared from the skin of a four-week old bull calf by the method of Piez et al. (1963). Phosphate reprecipitation (Gross, 1958) was carried out once,

Preparation No. 2. Acid-soluble collagen was prepared from the skin of a one-week-premature heifer calf as in preparation No. 1.

Preparation No. 3. Acid-soluble collagen was prepared from the skin of a one-week premature bull calf by the method of Steven and Tristram (1962). Phosphate reprecipitation (Gross, 1958) was not carried out.

In all these preparations neutral-salt-soluble collagen was exhaustively extracted from the skin prior to preparing the acid-soluble collagen. Extraction details are presented in Chapter II.

Column Chromatography.

Method (a) : The method was similar to that of Piez et al. (1963), using buffers prepared from the same starting buffer (pH 4.8 and I = 0.06) by the appropriate addition of NaCl. For gradient elution, 500 ml of I = 0.06 buffer and 500 ml of I = 0.16 buffer were used to elute the α - and β - subunits which required a total volume of 740 ml. Elution was then extended to 1000 ml using I = 0.26 buffer. The column (2.5 cm x 26 cm) was packed with CM-cellulose (lot No. 4146 ; BioRad Laboratories), which was regenerated between runs by the method of the above authors. The effluent fractions were collected in 10 ml aliquots, the fraction collector being kept at about 15°. The column effluent was monitored continuously at 230 mμ using a Beckman model DB spectrophotometer and a 1 cm flow cell. Collagen solutions were prepared in I = 0.06 acetate buffer (2 mg/ml), and denatured at 45° for 30 min. Solutions containing 40-50 mg of denatured collagen were run onto the column and eluted at a flow rate of 120 ml/hr using a Buchler micro-pump. The column was maintained at 40°.

Method (b) : But for the elution procedure and the CM-cellulose, the method was similar to method (a). The system of elution entailed the changing of buffers of increasing ionic strength using a liquid switch operated from a time clock. Using the same starting buffer (I = 0.06), three additional buffers were made up by the appropriate addition of NaCl. The ionic strength of these buffers corresponded to 0.10, 0.16 and 0.26 respectively. Buffer changes were made as follows:

0 to 840 ml eluted with I = 0.06 buffer;
840 to 1900 ml eluted with I = 0.10 buffer;
1900 to 2900 ml eluted with I = 0.16 buffer;
2900 to 5000 ml eluted with I = 0.26 buffer.

The column (4.3 cm x 46 cm) was packed with CM-cellulose (lot no. 17016 ; Schleicher and Schüll) and kept at 40°. Solutions of collagen, prepared and denatured as before (100 - 1000 mg) were pumped onto the column and eluted at a flow rate of 160 ml/hr.

Gel-filtration.

Method (a) : Estimates of molecular weights were obtained by gel-filtration by the method of Whitaker (1963) and Andrews (1964). Using a column (52.5 cm x 2.5 cm) of Sephadex G200 (lot no. To 2510 ; Pharmacia, Uppsala, Sweden) maintained at 20°, whole or fractionated preparations were eluted at a flow rate of 30 ml/hr with sodium phosphate buffer (pH 5.8 ; I = 0.12). The optical absorption of the column effluent

was measured at 230 mμ using a Beckman model DB spectrophotometer and a 1 cm continuous flow cell. The column was eluted under pressure using an Accu-Flo pump. Collagen preparations containing 2-10 mg protein were prepared in 0.15 M-acetic acid (2-3 mg/ml) and denatured at 45° for 30 min before running onto the column. Samples of acid-soluble collagen (preparation No. 3) were also denatured at 50, 60 and 70° for 30 min respectively in order to assess thermal stability. A calibration curve was obtained with purified preparations of the following globular proteins : bovine plasma albumin and bovine γ-globulin (Armour Pharmaceutical Co., Eastbourne, Sussex), egg albumin, trypsin and pepsin (Nutrional Biochemicals Corp., Cleveland, Ohio, U.S.A.). A graph of elution volume against log (molecular weight) gave a linear relationship over the molecular weight range 20,000 - 160,000.

Method (b) : Because of the lengthy chromatography procedures used in isolating subunits and components and the possibility of thermal degradation taking place under the prescribed conditions, the thermal stability of acid-soluble collagen was investigated using gel-filtration. Using a column (131 cm x 1 cm) of Sephadex G 100 (lot no. To-7120 ; Pharmacia, Uppsala, Sweden) jacketed at 20°, thermally denatured acid-soluble collagen preparations were eluted at a flow rate of 19 ml/hr with sodium phosphate buffer (pH 5.8 ; I = 0.12). The optical absorption of the column effluent was measured

as in method (a). Collagen solutions containing approximately 10 mg of preparation No. 3 were prepared in 0.15 M-acetic acid (3 mg/ml) and denatured at 40° for ½, 26, 53, 94, 143 and 188 hr respectively before running onto the column. The column was eluted under pressure using an Accu-Flo pump.

Amino Acid Analysis.

The amino acid compositions of acid-soluble collagen (preparation No. 3), and the isolated collagen subunits and components were determined with a Beckman 120B amino acid analyzer, after hydrolysis with 6N-HCl under reflux for 24 hr. For these analyses subunits α_1 and α_2, and component A were prepared by rechromatography (see under results), while component B was isolated after one column separation on CM-cellulose. Insufficient of the minor components C and D were available for analysis.

Ultracentrifugation.

Thermally denatured acid-soluble collagen (preparation No. 3) and isolated collagen subunits and components were examined by sedimentation velocity at 35° in a Spinco model E ultracentrifuge. The freeze-dried collagen preparations were dissolved to give 0.40% (w/v) solutions in sodium formate buffer, pH 3.75 and I = 0.15 (Piez et al. 1963). These were denatured by heating at 40° just before

placing in the ultracentrifuge cell. All patterns reproduced in Fig. 10 were taken 80 and 120 min after reaching a speed of 56,100 rpm. For ultracentrifugation subunit α_1 was prepared by rechromatography (see under results), while subunit α_2 and components A and B were isolated after one column separation on CM-cellulose. The β_{12}-subunit was isolated on CM-cellulose using method (a). Insufficient of the minor components C and D were available for analysis.

Melting Curves.

(a) Melting curves for the original acid-soluble collagen (preparation No. 3) and the subunits α_1 and α_2, and components A and B were obtained by transferring cold solutions into a viscometer at 10°. The temperature was then raised in steps after equilibrating for 30 min at each temperature (von Hippel and Wong, 1963 b). All samples were made up in 0.15M- acetic acid (0.4 mg/ml). Because of solubility difficulties, the subunits and components were heated at 35° for 2-3 min and then allowed to stand for at least one week at 0-4° before centrifuging at 5° and 28,000 x g, and measuring their melting points.

(b) Melting curves for the original acid-soluble collagen (preparation No. 2) were also taken using the "30 minute method" as in (a) by measuring optical rotation at 365 mμ. Collagen preparations were made up in 0.15M- potassium acetate, pH 4.8 (1.3 mg/ml) and

transferred to a Perkin-Elmer model 141 polarimeter. A 10 mm jacketed polarimeter tube connected to a thermistatically controlled water bath was used. Because of solubility difficulties, the subunits α_1 and α_2 were heated at 35° for 2-3 min while the components A and B were heated at 40° for 5 min before centrifuging at 5° and 28,000 x g, and measuring their melting points after allowing to stand at 0-4° for at least one week.

Mutarotation Measurements.

Collagen-fold formation of the collagen subunits and components was followed by optical rotation measurements at 15 ± 0.07° in a 10 mm water-jacketed tube using a Perkin-Elmer model 141 polarimeter. Measurements were made at 589, 578, 546, 436, 365 mμ respectively. Subunit and component preparations were made up in 0.15M- potassium acetate, pH 4.8 (1.3 mg/ml). Because of solubility difficulties, subunits α_1 and α_2 were heated at 35° for 2-3 min while components A and B were heated at 40° for 5 min before allowing to stand at 0-4° for at least one week. The procedure for following helix formation was to heat the preparations at 45° for 15 min and then cool for 45 sec under tap water. A test sample was then immediately transferred to the polarimeter tube or viscometer kept at 15°, and readings taken at regular intervals for periods up to 48 hr.

Results.

Chromatography on CM- Cellulose.

Thermally denatured acid-soluble collagen was initially fractionated on a short column using a simple linear salt gradient and "Cellex" CM-cellulose (BioRad Laboratories). Preparations No. 1, 2 and 3 all gave similar typical elution patterns (Piez et al. 1963), although the recovery of the α_2- subunit was inexplicably high in all cases. Weak resolution of the α_3- subunit (Piez, 1964) was evident from the shape of the α_1 peak in some instances. Assuming similar absorption indices (Piez et al. 1963) the recovery of subunits resulted in approximately 21% α_1, 6% β_{11}, 12% β_{12} and 61% α_2 for both preparations No. 1 and 3 (Fig. 3). In all cases, further exhaustive elution at high ionic strength (I = 0.26) resulted in the elution of a small additional component. The nature of this component was not considered in the present study (see Chapter V).

Because of the conflicting evidence regarding the presence of additional components in soluble collagens (see introduction), and because of reported differences in the exchange characteristics of different grades of CM-cellulose (Tristram et al. 1965) a second chromatography procedure was investigated. After considerable experimentation involving buffer gradients, gradient buffer changes, column lengths and different grades of CM-cellulose, a new chromatography procedure was developed. In all, 26 individual experimental

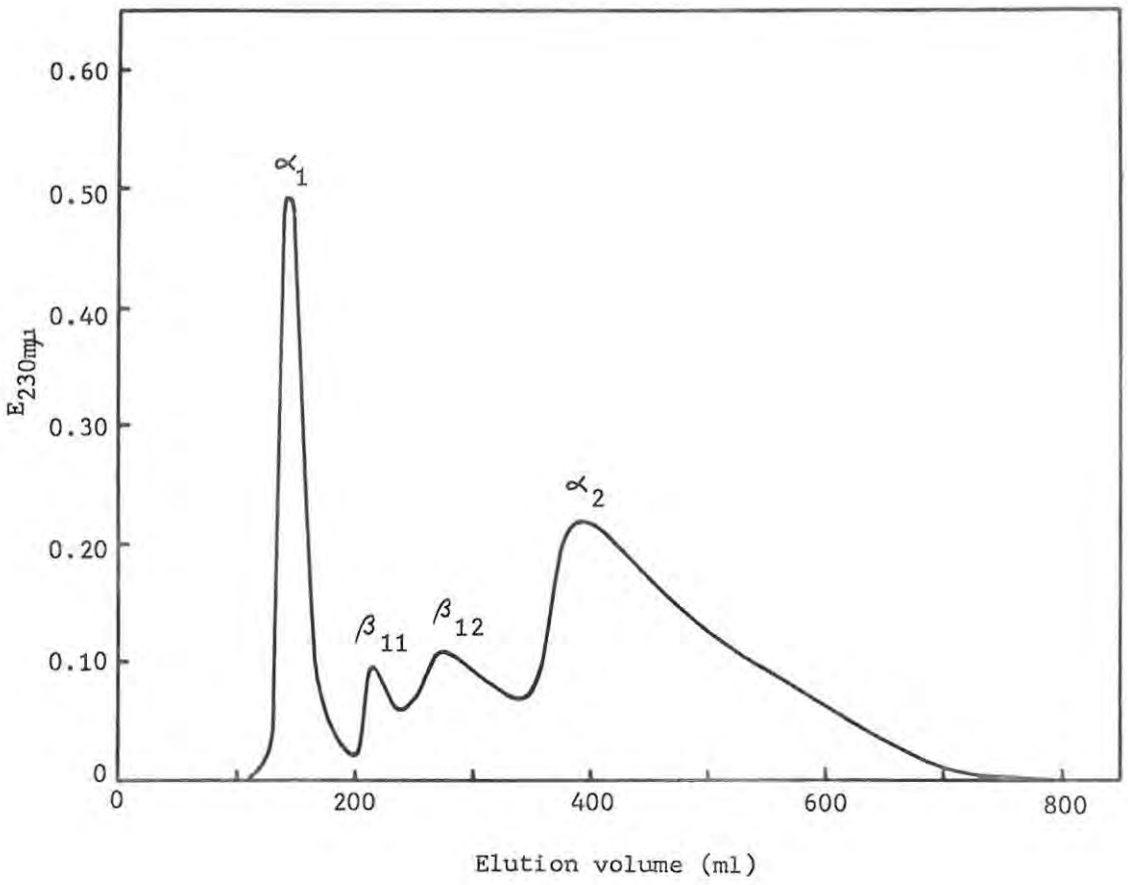

FIG. 3. Elution pattern of approximately 50 mg of acid-soluble calf-skin collagen on CM-cellulose at 40° after denaturation at 45° for 30 min. The column was eluted with a linear salt gradient using 500 ml. I = 0.06 acetate buffer and 500 ml. I = 0.16 acetate buffer.

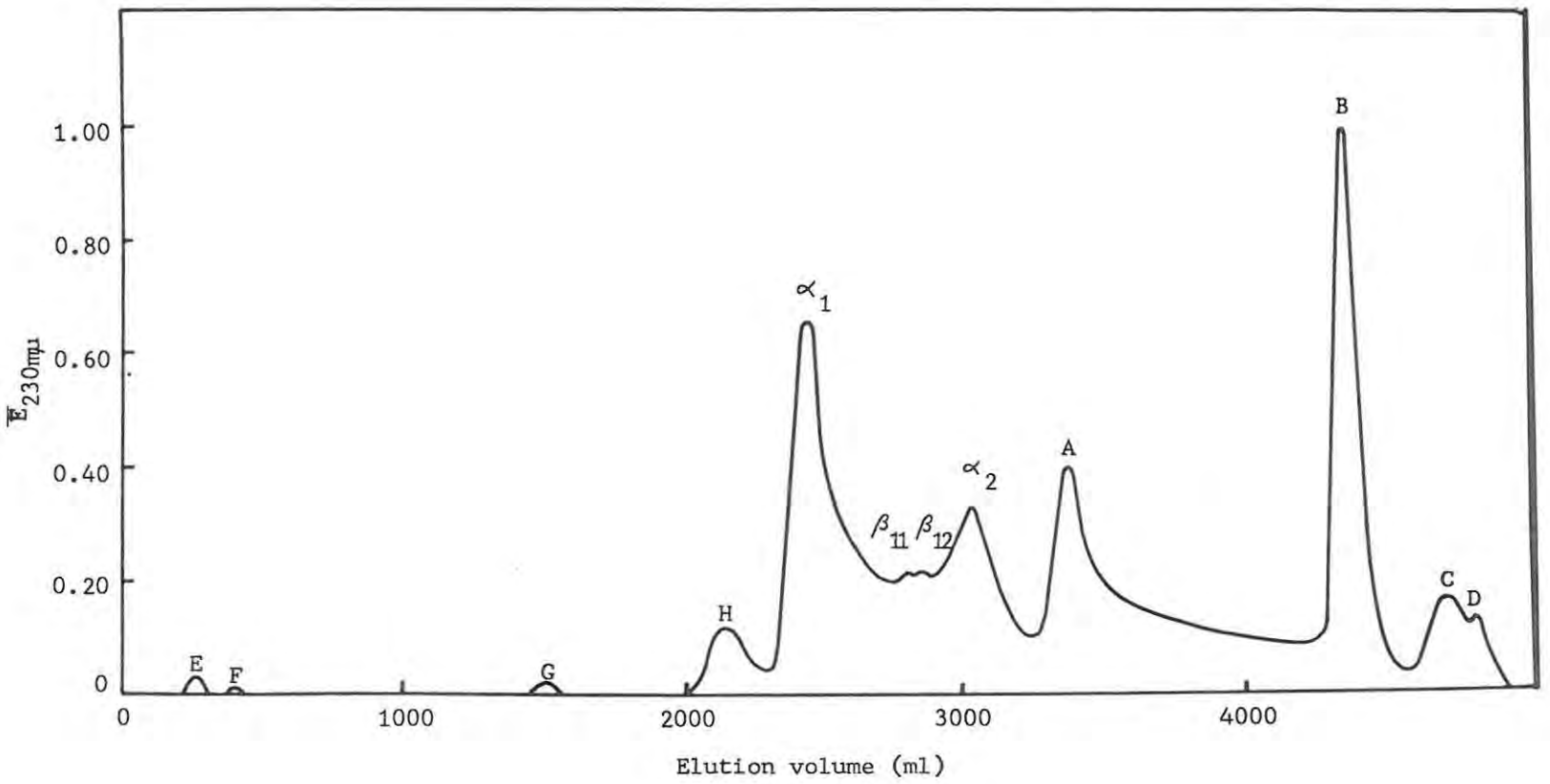

Fig. 4. Elution pattern of approximately 450 mg of acid-soluble calf-skin collagen on CM-cellulose at 40° after denaturation at 45° for 30 min. The column was eluted using changes of acetate buffers (pH 4.8) as follows: 0 to 840 ml eluted with I = 0.06 buffer; 840 to 1910 ml eluted with I = 0.10 buffer; 1910 to 2930 ml eluted with I = 0.16 buffer and 2930 to 5000 ml eluted with I = 0.26 buffer.

runs were carried out from which method (b) was evolved. The method entailed a more exhaustive elution procedure involving gradient buffer changes as well as a longer column containing a different grade of CM-cellulose (Schleicher and Schüll). Using this method, preparations Nos. 2 and 3 resulted in the separation of the α and β subunits together with four additional components designated A, B, C and D (Fig. 4). Exhaustive elution at high ionic strength (I = 0.26) resulted in no additional components.

In comparing the elution characteristics of the methods (a) and (b), it was noted that while the resolution of components A, B, C and D was not achieved using method (a), the resolution of β-subunits was likewise not satisfactory using method (b). The respective elution positions of the α_1- and α_2- subunits using method (b) were confirmed by the chromatography of these isolated subunits using method (a). It could only be concluded that the presence of the components A, B, C and D explained the large α_2 peak obtained using method (a). Further investigation was confined to the preparation and characterising these four additional components.

Originally 4 g of preparation No. 3 was fractionated using method (b) and the subunits α_1 and α_2, and components A and B isolated, exhaustively dialyzed against distilled water and weighed after freeze-drying. Components C and D were not recovered, as their presence was only detected at a later stage using a more exhaustive elution procedure (see below). The β - subunits were not considered because of incomplete

resolution. Because of the poor resolution of the α_2- subunit and component A, only limited amounts of pure α_2 and component A were isolated. The remaining material was collected as a composite (α_2 + A) preparation. Approximately 1600 mg of α_1, 300 mg of α_2, 300 mg of component A, 800 mg of α_2 + component A and 600 mg of component B were prepared in this way. The actual weight recoveries of subunits and components amounted to 80-90% of the original preparation fractionated in this way. Eleven chromatography runs on preparation No. 3 showed the high degree of reproducibility of the method, both in terms of elution volumes (Table I) and the quantity of subunits and components recovered. When compared with the method of Piez et al. (1963) in which only four subunits were resolved, the resolution of eight collagen fractions using method (b) has to be considered. While in some instances the resolution of components was not complete, these were further purified by rechromatography (see below).

The individual subunits α_1 and α_2 and components A, B as well as (α_2 + A) after preparation by chromatography, dialysis and freeze drying, were purified by further chromatography using method (b). For the purposes of this study, the latter procedure is defined as rechromatography. The individual subunits and components were then again isolated, dialyzed against distilled water, concentrated to half their original volume by suspending in a draught of air at 0-4^{o} and then lyophilised. The results of this rechromatography are

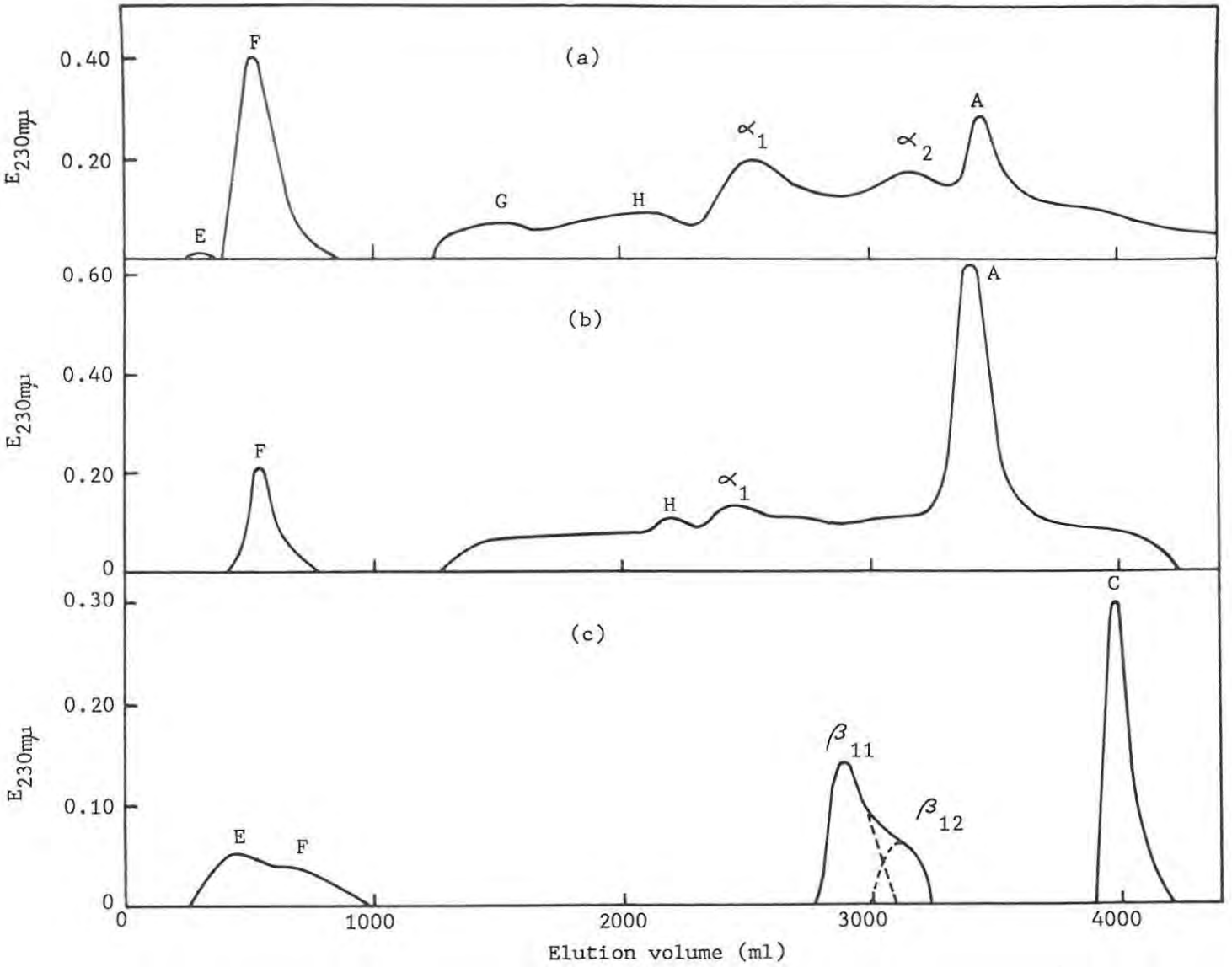

FIG. 6. Elution patterns of (a) 800 mg component A; (b) 500 mg α_2-subunit; (c) 7 mg components (C + D); on CM-cellulose at 40° after heating at 45° for 30 min. The columns were eluted using changes of acetate buffers (pH 4.8) as follows: 0 to 840 ml eluted with I = 0.06 buffer; 840 to 1910 ml eluted with I = 0.10 buffer; 1910 to 2930 ml eluted with I = 0.16 buffer and 2930 to 4660 ml eluted with I = 0.26 buffer.

recorded in Figs. 5 and 6. It was evident that large losses in the actual weight recovery of individual subunits and components occurred, especially in the case of the α_2 subunit and component B (Table II). This was later attributed to the precipitation of protein on the walls of the dialysis membrane when using distilled water as a dialysis medium. 0.15M-acetic acid was subsequently used for this purpose.

Assuming similar absorption indices (Piez et al. 1963), the compositions of the individual preparations were calculated from their respective chromatograms (Table III). It may be noted that the rechromatography of subunit α_1 yielded 13% of a component eluting in the position of component B, and conversely the rechromatography of component B yielded 71% of a component eluting in the α_1 position. Similarly the rechromatography of α_2 yielded 47% of a component eluting in the A position, and conversely the rechromatography of component A yielded 15% of a component eluting in the α_2 position. Also, the rechromatography of both α_2 and component A yeilded a fairly high percentage of material eluting in the E, F, G and H positions, as well as considerable amounts of material eluting in the α_1 position (Table III, Figs 5 and 6); indicating that both α_2 and A are relatively unstable at 40°.

Using a more exhaustive elution procedure, $1\frac{1}{2}$ g of preparation No. 2 was fractionated using this method and components (C + D) isolated, dialyzed against 0.15M-acetic acid and freeze-dried. The rechromatography

TABLE II.

EXPERIMENTAL WEIGHT RECOVERIES OF COLLAGEN SUBUNITS AND COMPONENTS AFTER RECHROMATOGRAPHY ON CM-CELLULOSE AT 40°.

Preparation	Original Weight before chromatography (mg)	Weight Recovered after chromatography (mg)	% Weight Recovery.
Rechromatography of α_1- subunit	1600	760	47.5
Rechromatography of composite (α_2+A)	800	56	7
Rechromatography of α_2 -subunit	300	28	7
Rechromatography of component A	300	104	34.5
Rechromatography of component B	600	2	0.3

TABLE III.

SUBUNIT COMPOSITION OF THERMALLY DEGRADED ACID-SOLUBLE CALF-SKIN COLLAGEN AFTER CHROMATOGRAPHY ON CM-CELLULOSE AT 40°.

Preparation	% Recovery of Subunits and Components.										
	E	F	G+H	α_1	β_{11}	β_{12}	α_2	A	B	C	D
Original Preparation			2	30	4[a]	3[a]	17	13	24	5	2
Rechromatography of subunit α_1	2	2	2	57			11	9	13		
Rechromatography of subunit α_2		10	10	18			10	47	2		
Rechromatography of component A	14	6	13	21			15	26	6		
Rechromatography of component B	1	5	2	71			18	10	5		
Rechromatography of components C+D	10[a]	5[a]			26[a]	12[a]				40	

[a] Due to incomplete resolution these values are only an approximation.

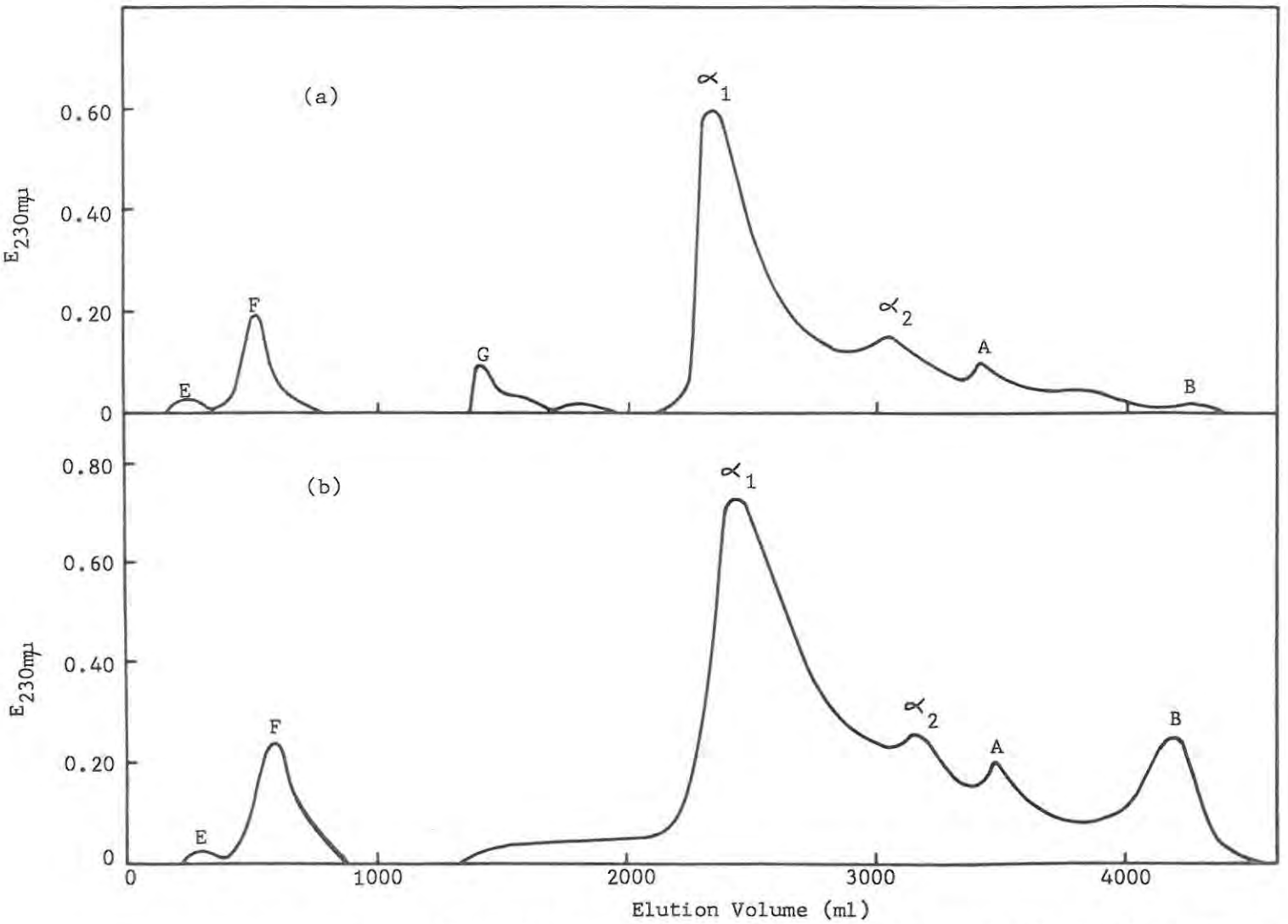

FIG. 5. Elution patterns of (a) 500 mg component B; (b) 800 mg α_1 - subunit; on CM-cellulose at 40° after heating at 45° for 30 min. The columns were eluted using changes of acetate buffers (pH 4.8) as follows: 0 to 840 ml eluted with I = 0.06 buffer; 840 to 1910 ml eluted with I = 0.10 buffer; 1910 to 2930 ml eluted with I = 0.16 buffer and 2930 to 4660 ml eluted with I = 0.26 buffer.

Table III after pg 43

of components (C + D) resulted in the elution of about 26% of a component eluting in the β_{11} position, about 12% in the β_{12} position; 40% in the position of the C component, as well as about 15% in the E-F position (Table III, Fig 6). Component D appeared to be completely unstable under the prescribed conditions.

The nature of the material eluting in the E, F, G and H positions has not been considered in the present study. A relation with the leading component E reported by other workers would appear to be evident (Schleyer, 1962 ; Piez et al. 1963).

It was initially thought that either the freeze drying of the original preparations (Tristram et al. 1965) or the overloading of test material on the column may have been responsible in some way for the presence of these additional components. Freshly prepared unfrozen preparations (Nos. 1 and 2) were thus fractionated, while sample loading in the range 100-1000 mg was investigated. In both cases, the resolution of additional components remained unchanged.

A total of 20 chromatography runs using method (b) showed good reproducibility although the elution positions of components B, C and D were not always consistant due to the extremely longthy elution procedure, but were easily characterised by their sequence and relative difference in size. Similarly, the rechromatography of the α_1- subunit repeated three times likewise gave good re-

producibility. Although the chromatographic method did not give complete resolution of adjacent components, thus facilitating the collection of relatively pure fractions, the suggested interconversions nevertheless involve chromatographic components which are in most cases widely separated in the elution sequence (e.g. the α_1- subunit and component B, see Figs. 5 and 6).

From these results it is strongly suggested that an interconversion or equilibrium between subunits and components takes place under the prescribed conditions. A relationship between α_1 and component B, α_2 and component A, β_{11} and component C and between β_{12} and component D is apparent. Such a relationship is also suggested by the ratio of the subunits and components recovered. The chromatography of preparation No. 3 resulted in a rather consistent ratio $\alpha_1/B = \alpha_2/A$ while similarly preparation No. 2 resulted in the ratio $\alpha_1/B = \alpha_2/A = \beta_{11} + \beta_{12}/C + D$. Tristram et al. (1965) also report an interconversion of their components 3 and 4 at temperatures in excess of the melting point of the acid-soluble collagen.

Gel-filtration on Sephadex.

Gel-filtration was used in the present study in order to assess the molecular weights of the fractionated collagen subunits and components, as well as to investigate the amount of thermal

degradation taking place during the preparation of these fractions. Because of the labile nature of these fractionated subunits and components prepared chromatographically at 40° (viz. α_2, A and D), gel-filtration through Sephadex G200 was carried out at 20°. The lengthy elution procedure using Sephadex G200 (9-10 hr) made the use of higher operating temperatures unsatisfactory. Under these conditions several sources of error are possible with the gel-filtration method. Firstly, the occurence of collagen-fold formation at 20° (Hippel and Wong, 1962) and secondly, the aggregation of subunits at this temperature (Boedtker and Doty, 1954 ; Engel, 1962). These were partially, if not wholly, overcome by heating the subunits and components at 40° prior to running onto the column, so converting helical structures to the random-coil form while also dissipating any aggregated subunits. Finally, while it was only possible to standardize the method using globular proteins, the present study was confined to the elution characteristics of thermally denatured collagen fractions. A difference in molecular shape may be anticipated. For such reasons the estimation of molecular weights of subunits and components using this method was found to give extremely high values. Nevertheless the method did indicate relative molecular weights which were of interest.

The original preparation No. 3 after thermal denaturation at 45° was thus separated into one large high molecular weight composite

component and one small low molecular weight fraction (Fig. 7). Similar elution patterns have been reported for thermally denatured soluble collagens (Francois and Glimcher, 1965 ; Piez, 1965 ; Blumenfeld at al. 1965). The major composite peak corresponded to the subunits and components, while the small peak eluting at about 270 ml represented the acetic acid used in dissolving the test sample. The effect of prolonged heating at 40° of the original acid-soluble collagen prior to gel-filtration using method (b) suggested very little degradation yielding low molecular weight products. Samples of acid-soluble collagen were heated at 40° in this way for 26, 53, 94, 143 and 188 hr respectively. Only the sample heated for 188 hr showed signs of molecular degradation (Fig. 8). In much the same way, samples of acid-soluble collagen were heated at 50, 60 and 70° respectively for 30 min prior to gel-filtration using method (a). The resulting elution patterns were similar in all cases to the pattern resulting from heating at 45° (Fig. 7). Thus, within the temperature limits prescribed, very little molecular degradation yielding low molecular weight material was indicated.

The gel-filtration of the α_1- subunit and the composite (α_2 + A) (see chromatography), both prepared by rechromatography resulted in the resolution of only a single well-defined high molecular weight peak (Fig. 9 (a) and (c)). On the other hand, both components A and B, prepared by rechromatography and by a single column separation

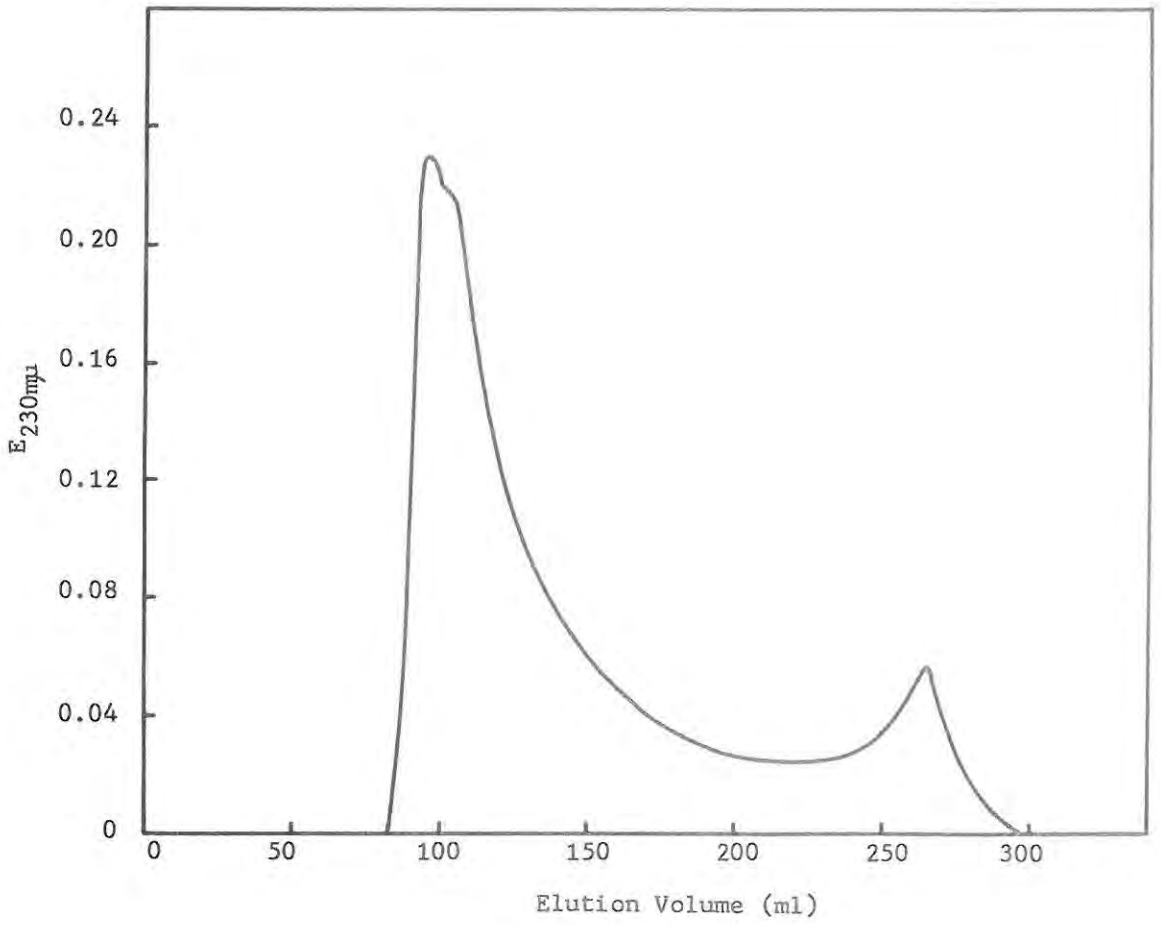

FIG. 7. Gel-filtration on Sephadex G200 at 20° of thermally degraded acid-soluble calf-skin collagen. 10 mg of soluble collagen in 3 ml 0.15 M - HAc was heated at 40° for 30 min and eluted with phosphate buffer (pH 5.8; I = 0.12).

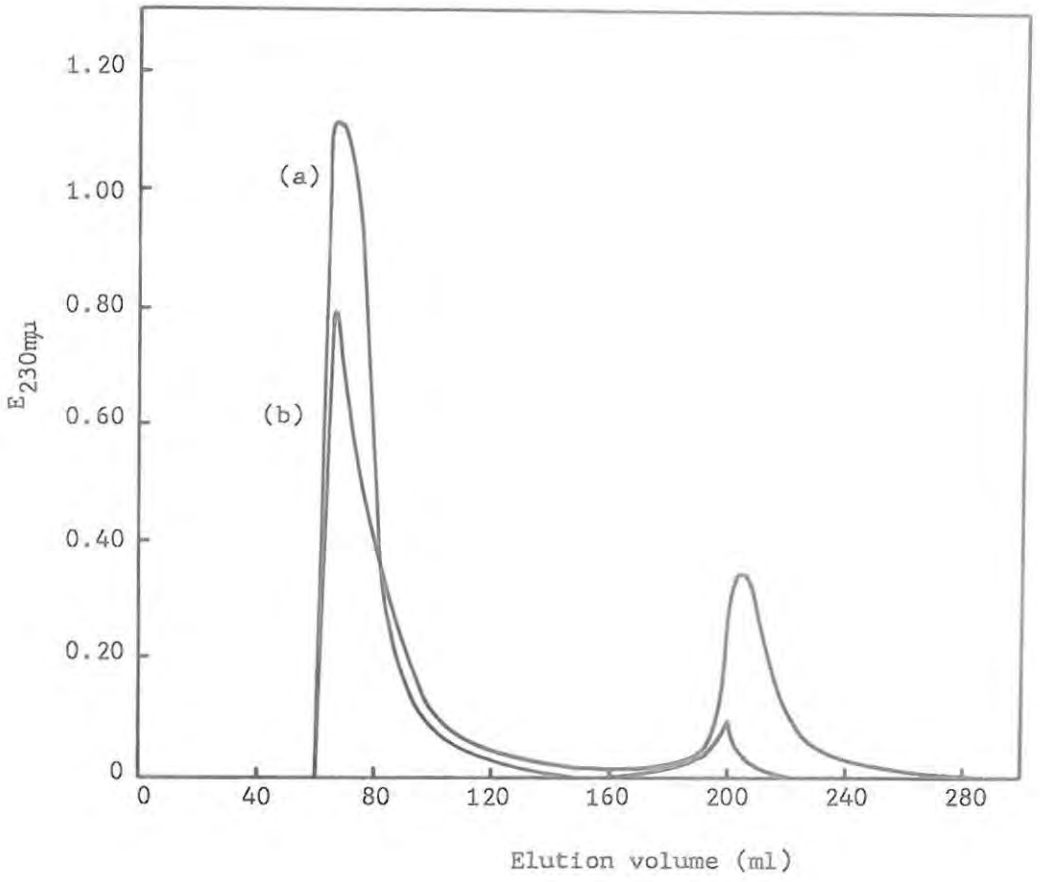

FIG. 8. Gel-filtration on Sephadex G100 at 20° of thermally degraded acid-soluble calf-skin collagen. 10 mg of soluble collagen in 3 ml 0.15 M - HAc was heated at 40° and eluted with phosphate buffer (pH 5.8; I = 0.12).
(a) Thermally degraded at 40° for ½ hr;
(b) Thermally degraded at 40° for 188 hr.

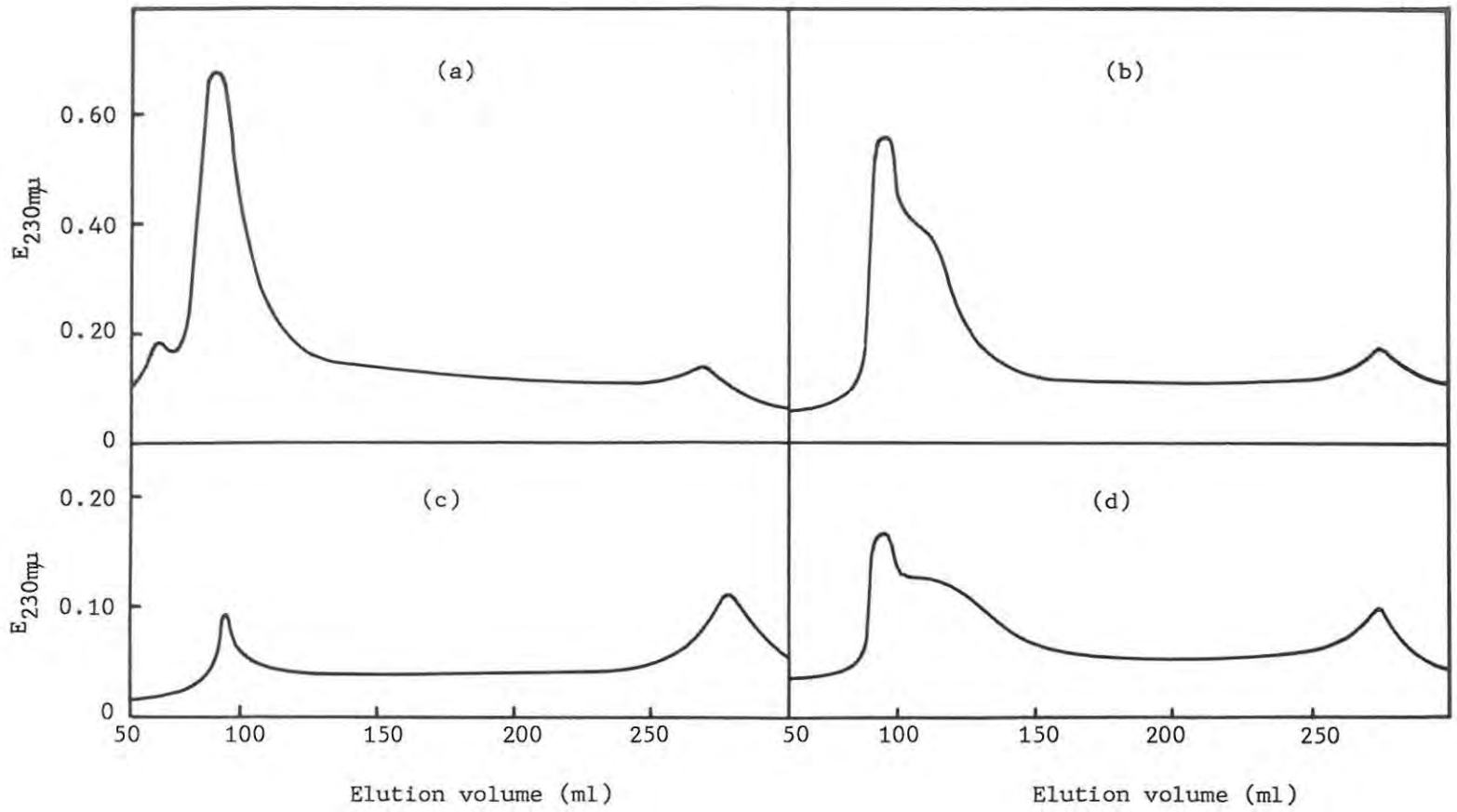

FIG. 9. Gel-filtration on Sephadex G200 at 20° of thermally degraded collagen subunits and components (a) α_1 - subunit; (b) component B; (c) α_2 - subunit + component A; (d) component A. The isolated fractions were heated at 40° for 30 min and eluted with phosphate buffer (pH 5.8 and I = 0.12).

respectively, resulted in the resolution of a composite high molecular weight peak (Fig. 9 (b) and (d)). Furthermore, when allowing for the acetic acid used in dissolving the test samples, considerable amounts of low molecular weight material were eluted in the case of both components (α_2 + A) and component A. This would support the evidence from the rechromatography of the α_2-subunit and component A, viz. that these components are both relatively unstable under the prescribed conditions of rechromatography.

Amino Acid Analysis.

The amino acid composition of preparation No. 3 and subunits α_1 and α_2, and components A and B are reported in Table IV. These analyses show that subunit α_1 and component B are closely related, but differ significantly from α_2 and component A which in turn appear similar. Component B and α_1 contain more hydroxyproline and methionine, but less tyrosine and hydroxylysine than the parent collagen. Component A and α_2 contain more arginine, aspartic acid, histidine, hydroxyproline, isoleucine, leucine, phenylalanine and valine, but less lysine and tyrosine than the parent collagen. The analyses of the subunits α_1 and α_2, and components A and B are compared with the original acid-soluble collagen in Table V. The excess of cationic residues in the subunits and components of acid-

TABLE IV.

AMINO ACID COMPOSITION OF ACID-SOLUBLE CALF-SKIN COLLAGEN AND ITS CONSTITUENT SUBUNITS AND COMPONENTS.

Amino Acid	Amino Acid Composition[a] (g amino acid residue/100 g of protein) Original Preparation	α_1	α_2	A	B
Ala	9.13	9.06	8.55	8.48	8.72
Arg	7.35	8.81	8.20	8.60	7.93
Asp	5.61	5.69	6.48	6.89	6.12
Cys	Nil	Nil	Nil	Nil	Nil
Glu	10.06	10.42	10.50	10.08	9.93
Gly	21.22	21.09	21.67	21.11	19.92
His	0.48	0.47	0.73	0.89	0.49
Hyl	1.15	0.86	0.98	1.35	0.91
Hyp	11.15	12.32	12.48	14.09	14.78
Ile	1.33	1.18	1.72	1.80	1.27
Leu	2.87	2.64	3.40	3.61	2.75
Lys	3.40	3.51	2.92	2.73	3.32
Met	0.59	0.83	0.21	0.60	0.73
Phe	1.87	1.90	2.18	2.12	1.93
Pro	12.80	13.25	12.52	11.91	12.18
Ser	3.27	3.26	3.42	3.43	3.27
Thr	1.73	1.84	1.90	1.91	1.83
Tyr	0.51	0.39	0.41	0.42	0.39
Val	2.05	1.81	2.84	2.97	1.94
Amide N	(0.77)	-	-	-	-
TOTAL	96.57	99.33	101.08	102.99	98.41
TOTAL N	18.3	-	-	-	-

[a] The results for acid-soluble collagen and subunit α_1 are based on duplicate analyses, and those for subunit α_2 and components A and B on a single analysis.

TABLE V.

A COMPARISON OF THE AMINO ACID ANALYSES OF THE SUBUNITS AND COMPONENTS OF ACID-SOLUBLE COLLAGEN WITH THE PARENT MATERIAL.

Amino Acid	± % Difference			
	α_1- Subunit	α_2- Subunit	Component A	Component B
Arg	+19.8	+11.6	+17.0	
Asp		+15.5	+22.8	
His		+52.1	+85.5	
Hyl	-25.2		+17.4	-20.8
Hyp	+10.5	+11.9	+26.4	+32.6
Ile		+29.3	+35.3	
Leu		+18.5	+25.8	
Lys		-14.1	-19.7	
Met	+40.7	-64.5		+23.7
Phe		+16.6	+13.4	
Tyr	-23.5	-19.6	-17.6	-23.5
Val		+38.5	+44.9	

soluble-collagen is presented in Table VI. Both tables V and VI illustrate the similarities between α_1 and component B, and between α_2 and component A.

But for higher tyrosine contents, the analyses for the α_1- and α_2- subunits compare favourably with the α_1 and α_2 values reported in earlier findings. (Piez et al. 1963 ; Piez, 1965 ; Drake et al. 1966).

Ultracentrifugal Analysis.

The sedimentation coefficients for thermally denatured acid-soluble calf-skin collagen (preparation No. 3) together with the α_1-, β_{12}-, and α_2- subunits and components A and B are presented in Table VII. While the sedimentation coefficients have not been extrapolated to infinite dilution, ruling out any determination of molecular weights, both the experimental procedure and the gelatin concentrations were similar to those of Piez et al. (1963). A correlation with these results may thus be made.

Both the α_1- and α_2- subunits appear to be homodisperse fractions with sedimentation coefficients $S_{20,w} = 2.5$ (Fig.10). These values compare favourably with those of Piez et al. (1963). Components A and B both result in the resolution of two peaks having sedimentation coefficients similar to the α - and β - subunits respectively (Fig. 10 and Table VII). The sedimentation pattern of

TABLE VI.

EXCESS OF CATIONIC RESIDUES (per 1000 total residues) IN THE SUBUNITS AND COMPONENTS OF ACID-SOLUBLE CALF-SKIN COLLAGEN AT pH 4.8

Anionic Residues	α_1	α_2	A	B
Glu	80.7	81.3	78.1	76.9
Asp	49.4	56.3	59.9	53.2
	130.1	137.6	138.0	130.1
assuming 86.3% ionisation*	112.4	118.8	119.1	112.4
less Amide N#	41	41	41	41
TOTAL	71.4	77.8	78.1	71.4
Cationic Residues.				
Lys	27.4	22.8	21.3	25.9
Hist	3.4	5.3	6.5	3.6
Arg	56.5	52.5	55.1	50.8
Hyl	6.0	6.8	9.4	6.3
TOTAL	93.3	87.4	92.3	86.6
less anionic total	71.4	77.8	78.1	71.4
	21.9	9.6	14.2	15.2

* Tristram et al. 1965.

\# Piez et al. 1963.

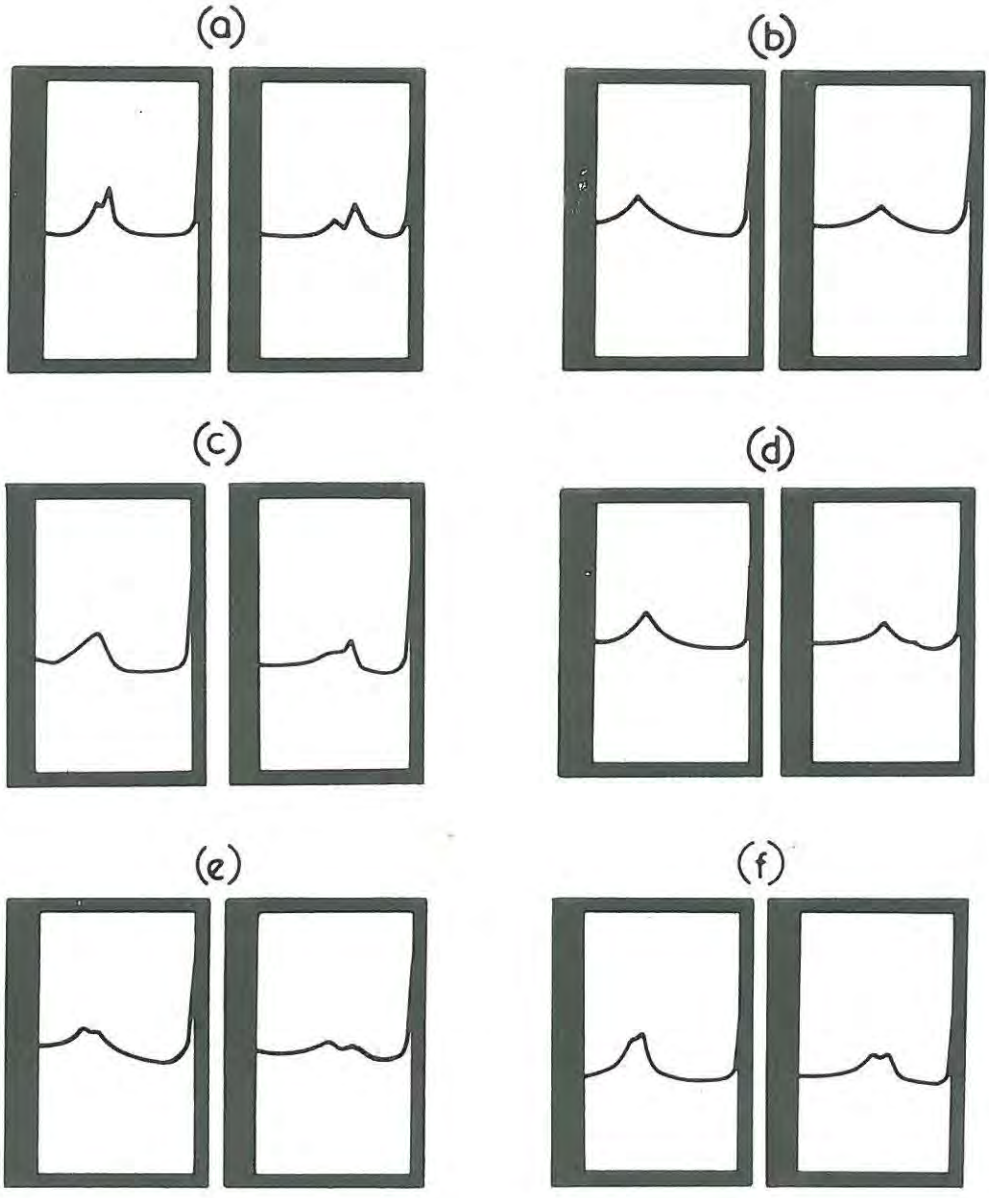

FIG. 10. Sedimentation patterns of (a) thermally denatured acid-soluble calf-skin collagen; (b) α_1 - subunit; (c) β_{12} - subunit; (d) α_2 - subunit; (e) component A; (f) component B. The photographs were taken after 80 and 120 min at 56,100 r.p.m. and 35°. The solutions contained 0.4% (w/v) of protein in sodium formate buffer (pH 3.75 and I = 0.15). Sedimentation is from left to right.

TABLE VII.

ULTRACENTRIFUGAL ANALYSIS OF ACID-SOLUBLE CALF-SKIN COLLAGEN AND ITS CONSTITUENT COMPONENTS[a]

Material	$S_{20,w}$ (S)
Acid-soluble collagen	2.6, 3.4
Subunit α_1	2.5
Subunit β_{12}	3.0
Subunit α_2	2.5
Component A	2.8, 3.4
Component B	2.5, 3.4

[a] The sedimentation coefficients were measured at 56,100 rpm and 35°, and at the same concentration, but are uncorrected for concentration-dependence.

the thermally denatured parent material (preparation No. 1 and 3) resulted in two major peaks with sedimentation coefficients of 2.6 and 3.4 respectively (Fig. 10 and Table VII). These major components were assumed to correspond to the α- and β- subunits (Piez et al. 1963). The measurement of areas under the peaks (preparation No. 3) resulted in approximately 47% α and 53% β, although the Johnston-Ogston effect (Johnston and Ogston, 1946) would result in a slight over estimation of the β - subunit.

Since the chromatography of the original thermally denatured preparation No. 3 using method (b) resulted in the recovery of subunits α_1- and α_2- in excess of 47% (Table II), while method (a) indicated a β content of only 18%, a molecular weight similar to the β - subunit is suggested for the major components A and B. This suggestion is supported by the presence of the β - like moeity present in both components A and B.

No γ - like component was evident in either preparations No. 1 or 3.

Because the present study was concerned with the possible thermal degradation of both subunits and components under the prescribed conditions of chromatographic separation at 40°, the thermal stability of these collagen fractions was also investigated using sedimentation. Samples of acid-soluble collagen (preparation

No. 2) were heated for 30 min at 45, 50 and 60° respectively before ultracentrifugation at 35°. The resulting sedimentation patterns were all similar to the effect of heating at 40° (see method and Fig. 10), indicating no degradation of either subunits or components under such conditions.

Melting Curves.

Since information can be obtained about the helical properties of collagen from melting curves, these were initially measured by viscosity. von Hippel and Wong (1963a) have shown that the melting curves of collagen are very similar when measured by changes in optical rotation or viscosity by the non-equilibrium "30 minute-method", as used in the present study. From the data in Fig. 11 it is possible to determine the melting point (Tm), which is the temperature of the mid-point of the transition, $\Delta \left(\frac{\eta_{sp,T}}{\eta_{sp,10^\circ}} \right)_T$ being the difference between the temperature corrected specific viscosity at the two ends of the transition, and ΔT the difference in temperature between the points at which the helix-coil transition is one-fourth and three-fourths complete. von Hippel and Wong (1963) have defined the latter, when measured by optical rotation, as being directly related to the sharpness of the phase transition, serving as a measure of the "degree of co-operativeness" of the helical structure. The results for acid-soluble collagen (preparation No. 3), subunits α_1

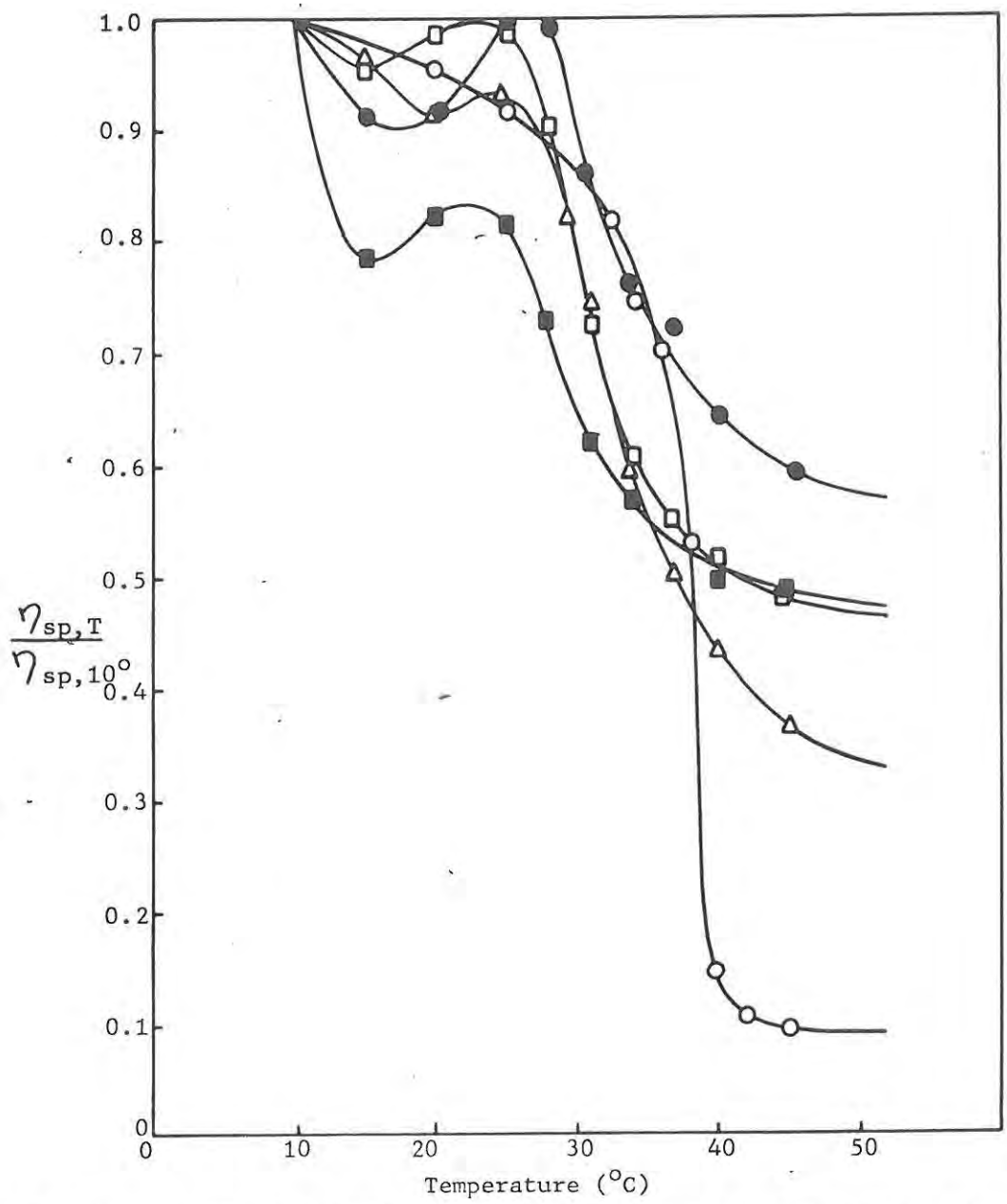

FIG. 11. Melting curves of: native acid-soluble calf-skin collagen O; α_1 - subunit □; α_2 - subunit ● ; component A Δ; component B ■. Sample solutions were kept at 0-4° for 7 days prior to melting. Concentrations were approximately 4.5 mg/ml in 0.15 M - HAc.

and α_2 and components A and B are given in Table VIII.

The Tm values for the α_1 and α_2 subunits and the A and B components are significantly higher than the values reported for the α_1-, α_2- and β_{12}- subunits from rat skin collagen, namely, 26.6°, 22.5° and 26.1° respectively (Piez and Carrillo, 1964), where rat skin collagen has a value Tm = 38.0° (Piez and Carrillo, 1964) and the acid-soluble collagen used in the present study a value Tm = 38.1°. This would appear to indicate a higher degree of crosslinking or association in these subunits and components.

The higher values for ΔT found for the subunits α_1 and α_2 and components A and B when compared with the acid-soluble collagen from which they were prepared (Table VIII), would indicate that these components have a less ordered structure than that of acid-soluble collagen ; with subunit α_1 having the most order. The sharpness of the phase transition has been described as being due to the order of the helical structure (von Hippel and Wong, 1936b).

Flory and Weaver (1960) have emphasised that another characteristic of the melting curves, is the temperature at which all the structure has been melted. They point out that this temperature, as measured by viscometry, was the same (about 41°) for native rat-tail-tendon collagen and cooled gelatin. On the other hand, Piez and Carrillo (1964) found that the residue optical rotation level for their

After 52

TABLE VIII.

PARAMETERS FOR THE COLLAGEN-FOLD TO GELATIN TRANSITION FOR ACID-SOLUBLE COLLAGEN, SUBUNITS α_1, AND α_2 AND COMPONENTS A AND B, AS MEASURED BY VISCOSITY CHANGES.

Material	T_m (°C)	ΔT (°C)	$\Delta\left(\frac{\eta_{sp,T}}{\eta_{sp,10}}\right)$
Acid-soluble collagen	38.1	4.7	0.90
Subunit α_1	30.9	6.0	0.55
Subunit α_2	32.8	8.6	0.44
Component A	32.5	8.2	0.69
Component B	30.8	10.7	0.52

α_1- , α_2- and β_{12}- subunits was reached at least 4° below that of the rat-skin from which they were prepared. They attribute this to the presence of high molecular weight aggregates which can renature to triple-chain structures. In contrast to both these findings, the melting curves (Fig. 11) show that, while the temperature at which the helical structure of acid-soluble collagen is completely melted is about 44°, the equivalent points are reached by both subunits α_1 and α_2 and components A and B at a much higher temperature of about 54°.

The melting curves (Fig. 11) display irregularities or shoulders, the α_2- subunit showing the most prominent deviations. von Hippel and Wong (1963a) attribute these irregularities to covalent interchain bonding.

Mutarotation at 15°.

The rate and extent of optical rotation and viscosity recovery of cooled solutions of parent gelatin, obtained by thermal denaturation of soluble collagen ; are directly related to the α -, β - and γ - subunit content of the parent gelatin (Piez and Carrillo, 1964), or in other words to the degree of intramolecular bonding present in the collagen. Accordingly, solutions of collagen subunits and components in 0.15M- potassium acetate buffer (pH 4.8) ; which is known to have the minimum salt effect on mutarotation (Piez and Carrillo,

1964) ; were heat denatured at 40° for 15 min and their mutarotation characteristics studied by optical rotation or viscosity procedures.

The recovery of optical rotation for the α_1- subunit using the Bellingham and Stanley polarimeter is recorded in Fig. 12 ; while the increase in reduced viscosity for the α_1- subunit is shown in Fig. 13. These results show that the mutarotation of the α_1- subunit, within the concentration range 0.37 to 1.28 mg/ml, appears to be independent of concentration. According to the conclusions of Piez and Carrillo (1964), this would indicate at least two cross-linked polypeptide chains capable of undergoing helix formation on cooling. Furthermore, the recovery in optical rotation in these experiments was of the order of 94% after only 48 hr of recovery time at 15°, and was in fact still increasing after this period (Fig. 12). Piez and Carrillo attribute such a high recovery to the γ-subunit only. In addition, the specific optical rotation of the α_1- subunit was $[\alpha]_D^{15} = -326$, compared with a value of $[\alpha]_D^{20} = -415$ for the native acid-soluble collagen from which it was prepared.

Similar experiments were carried out with the α_2- subunit, the results being presented in Fig. 14. The recovery in optical rotation after 48 hr at 15° was 85% of the value before thermal denaturation took place. In this case, however, the mutarotation appeared to be concentration dependent. A similar finding has been

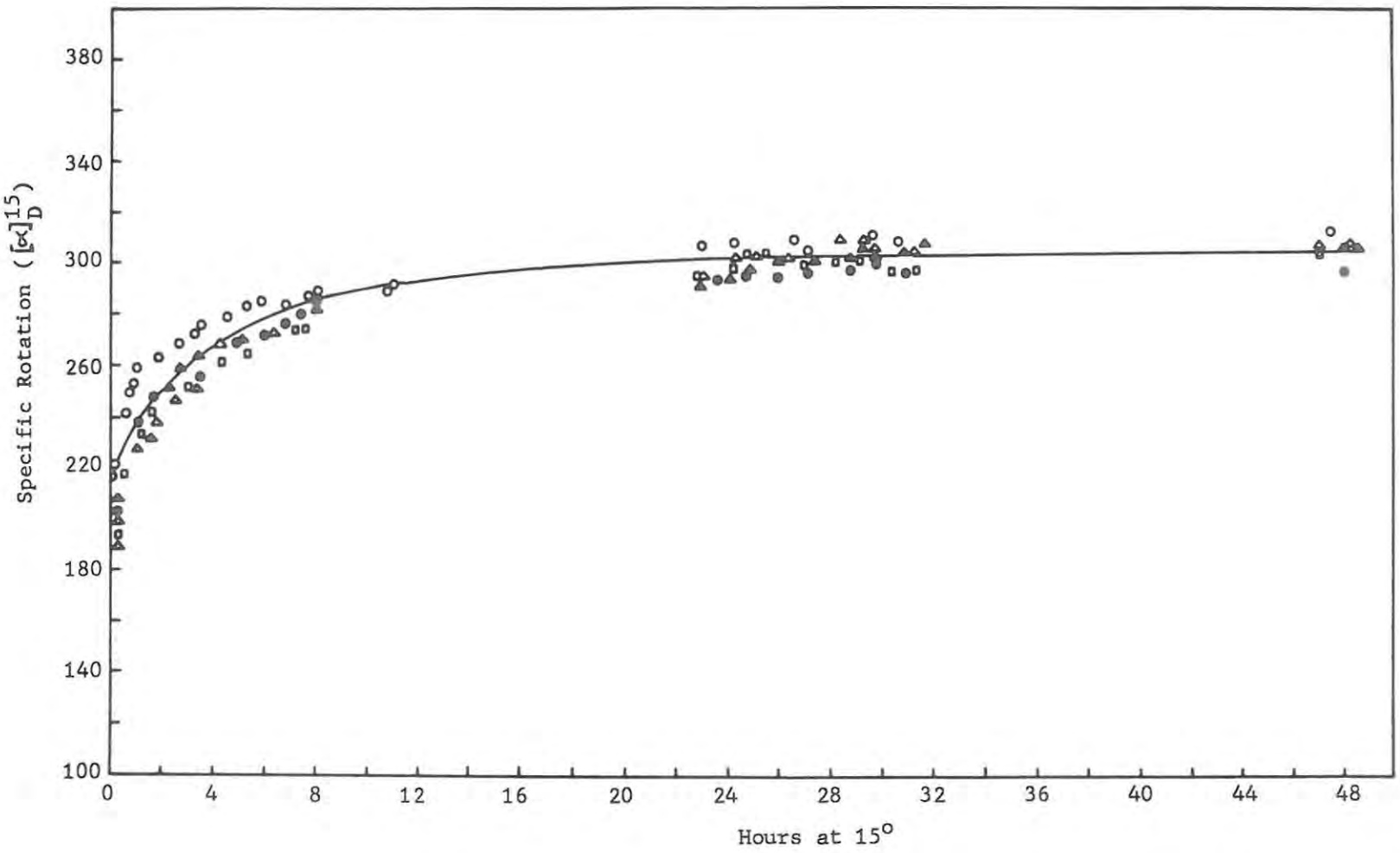

FIG. 12. Helix formation at 15° by the α_1 - subunit as measured by optical rotation : ●, 1.28 mg/ml; ○, 1.19 mg/ml; △, 0.83 mg/ml; ▲, 0.60 mg/ml; □, 0.37 mg/ml.

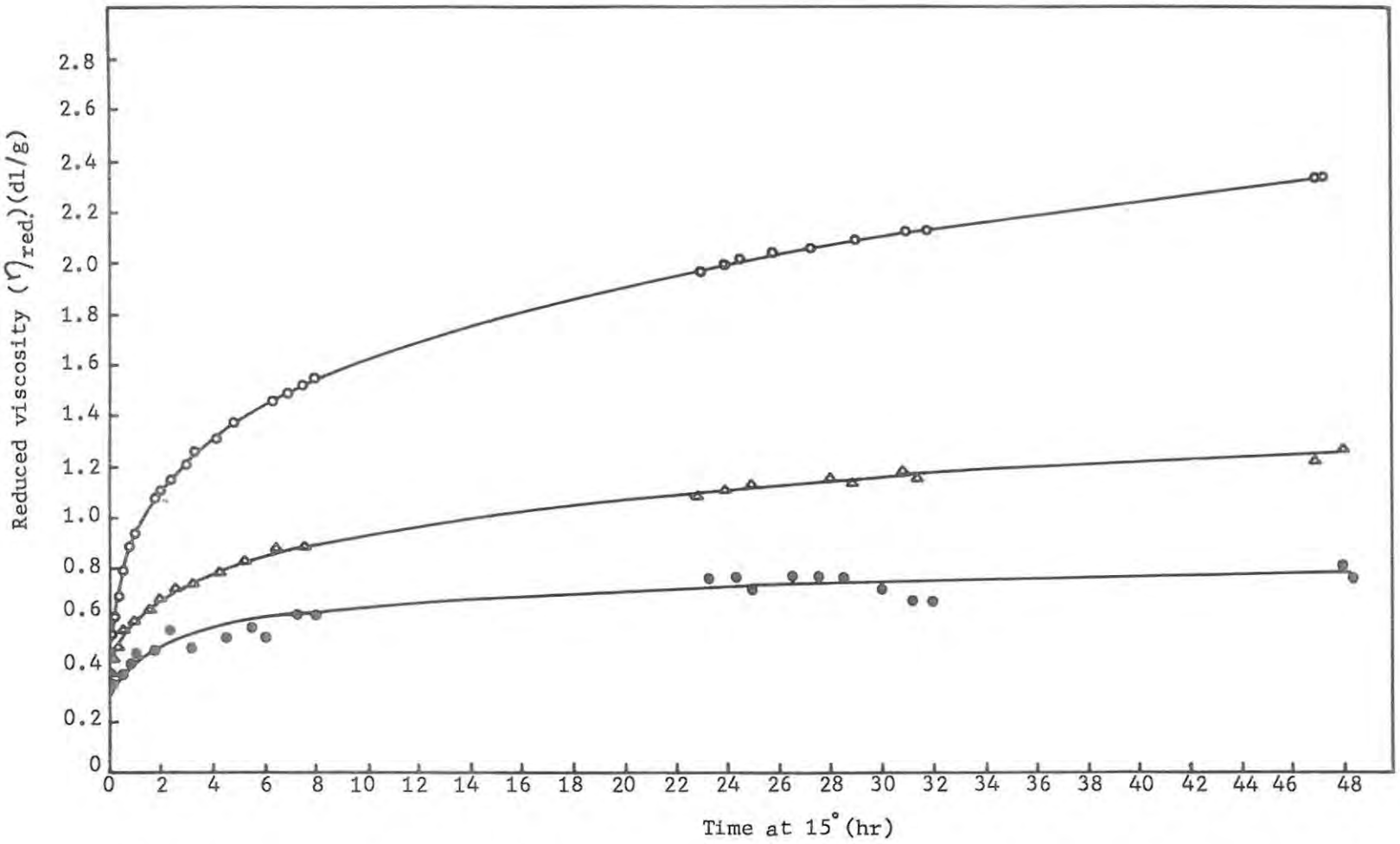

FIG. 13. Helix formation at 15° by the α_2 - subunit as measured by optical rotation : ○, 1.18 mg/ml; △, 0.80 mg/ml; ●, 0.59 mg/ml.

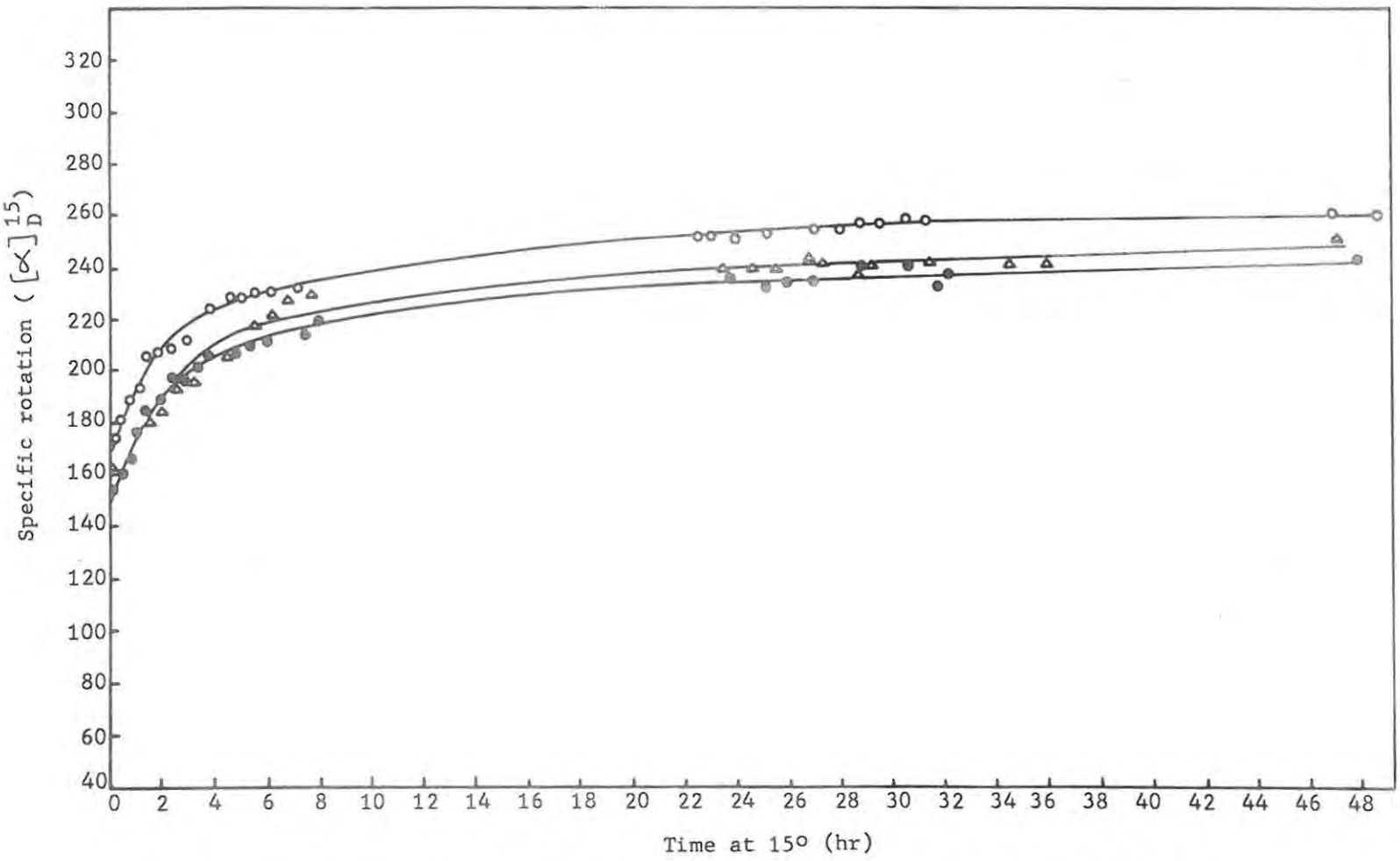

FIG. 14. Reduced viscosity of the α_1 - subunit during helix formation at 15°. O, 1.19 mg/ml: △ 0.60 mg/ml: ●, 0.37 mg/ml.

reported for the α - subunit by Piez and Carrillo, 1964. The specific rotation of the α_2- subunit ($[\alpha]_D^{15} = -311$) again corresponds to a component with a high degree of helical content, the value being only slightly lower than that for the α_1- subunit.

Piez and Carrillo (1964) attribute the pyrrolidine ring content, the subunit content and the solvent employed as being the prime factors that control the rate and extent of mutarotation in solutions of cooled gelatins. Harrington and von Hippel (1961) suggest a three stage mechanism : (1) The pyrrolidine-rich portions of the peptide chain undergo initial changes that nucleate the poly-L-proline II type helix. (2) This helix structure is propagated outwards along single gelatin chains (Flory and Weaver, 1960). (3) Lateral chain association through inter-chain hydrogen bonding is made possible by the helix formation of individual chains (Veis, 1964). The first stage may be followed by amino acid analyses, while the second and third stages may be monitored by optical rotation and viscosity respectively.

Because of the obvious discrepancies regarding the present findings and those reported by Piez and Carrillo (1964), the above experiments were repeated using the Perkin-Elmer model 141 polarimeter. The mutarotation characteristics of the parent acid-soluble collagen are compared with those of the α_1- and α_2- subunits and with those of components A and B (Fig. 15). The protein concentration was limited to 1.3 mg/ml in all cases.

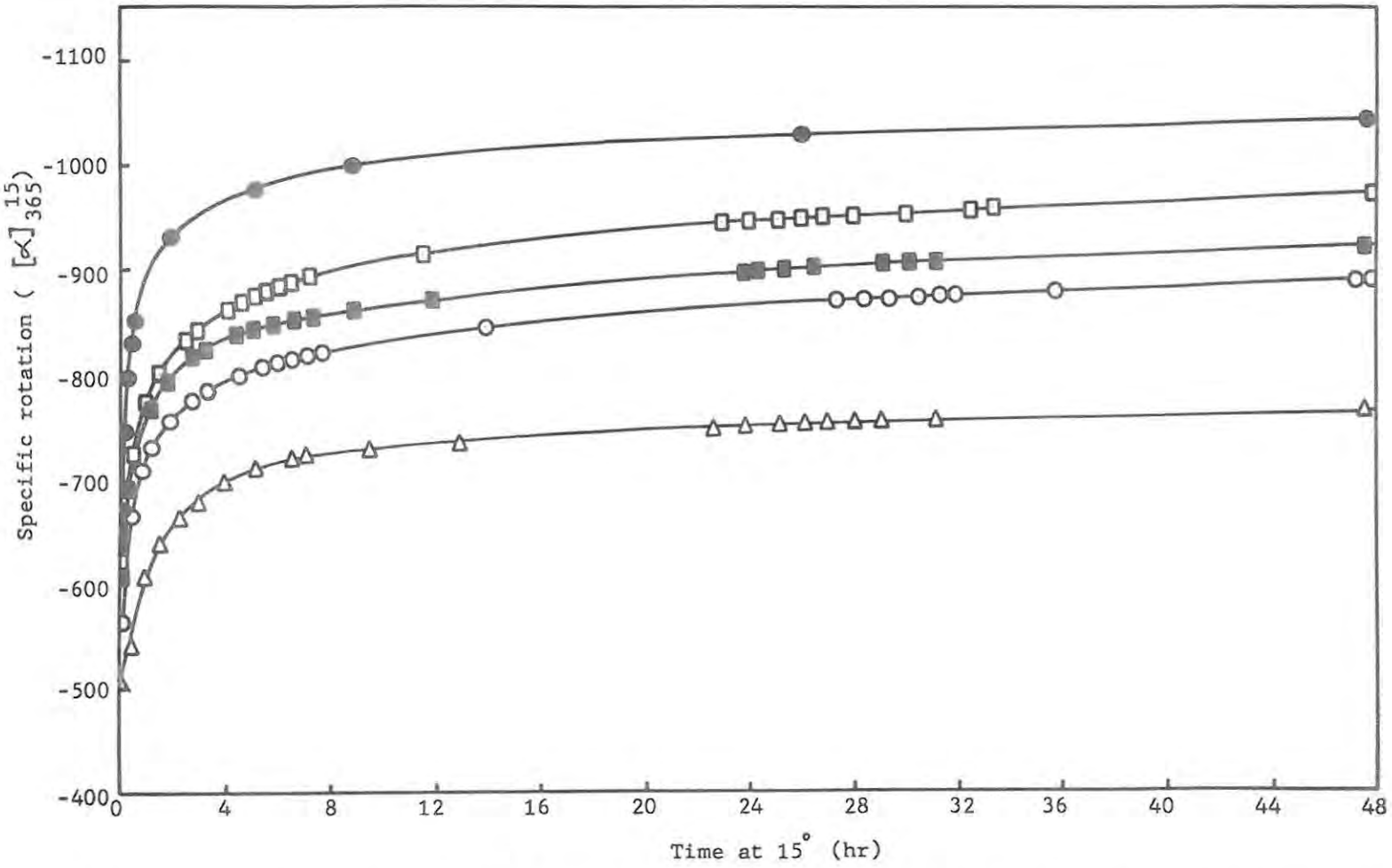

FIG. 15. Helix formation at 15° of thermally degraded acid-soluble calf-skin collagen and its subunits and components as measured by optical rotation. ●, acid-soluble collagen; □, α_1 - subunit; △, α_2 - subunit; ■, component A; ○, component B. Preparations were made up in 0.15 M-KAc (1.35 mg/ml).

Again, similar results with abnormally high recoveries indicative of the β - subunit (Piez and Carrillo, 1964) were obtained for both the α_1- and α_2- subunits. Recoveries of 89% and 73% after 48 hours at 15° for the α_1- and α_2- subunits were recorded respectively. Recoveries of 88% and 92% were recorded for the A and B components (Fig. 15).

As the identity of the present α_1- and α_2- subunits has been established, the observed differences in the mutarotation character as well as the melting curves of these subunits and components may only be attributed to differences in the original preparations. Piez et al. (1963, 1964) use repeated phosphate precipitation (Gross, 1958) in preparing acid-soluble collagen. Such an alkaline treatment may well depolymerise polymeric collagen (Courts, 1960), but at the same time damage or destroy the tyrosine rich telopeptides (Rubin et al. 1963). Since acid-soluble collagen was prepared using the method of Steven and Tristram (1962) in the present study, such a difference in preparations may be speculated. The high recoveries of tyrosine reported for this preparation when compared with the values reported by Piez et al. (1963) would support this arguement.

It may thus be speculated that the telopeptides play a vital role in the rate and extent of mutarotation in solutions of cooled gelatins as well as in the isolated subunits and components (see Chapters IV and V).

Discussion.

It has been shown that the chromatography of acid-soluble calf-skin collagen on thermal denaturation yields the subunits α_1, β_{11}, β_{12} and α_2, as well as the components A, B, C and D. These subunits and components together constitute at least 90% of the preparations studied. Using chromatography on CM- cellulose, Piez et al. (1963) recovered only α- and β- subunits from thermally denatured acid-soluble collagen. Schleyer (1962), Tristram et al. (1965) and the present author, using similar procedures, have shown the presence of these α- and β- subunits together with up to four additional components. The character of these additional components and their relationship to the parent material is at present not known.

The evidence from sedimentation would suggest that the major components A and B have molecular weights similar to the β- subunit. Furthermore, it is evident that these components are labile under the prescribed conditions, yielding material having a molecular weight similar to the α- subunit. This conclusion must be treated with caution, however, as differences in the molecular shape rather than size of these components may introduce conflicting sedimentation characteristics.

In their work on the separation of the subunits of calf-skin collagen, Piez et al. (1960, 1963) conclude that the elution sequence

α_1, β_{11}, β_{12}, α_2 is dependent upon charge density rather than molecular weights since it is known that the molecular weights of the two subunits differ approximately two-fold. Tristram et al. (1965) have calculated the excess of cationic residues from the analyses of Piez et al. (1960, 1963), assuming a pK COOH = 4.0 (i.e. 86.3% of carboxyl groups are dissociated at pH 4.8). Since the amino acid analyses (see results) indicate a similarity between α_1 and component B, and between α_2 and component A, a difference in molecular weight or shape can only explain the elution characteristics of these components. As the evidence from sedimentation suggested a molecular weight similar to the β - subunit, a difference in molecular shape from the intramolecularly bound β - subunit may be inferred. Under such conditions, components with higher axial ratios might be expected to elute more slowly than the β - subunits.

The rechromatography of both subunits and components at 40° was originally carried out as a preparative step prior to further physical and chemical characterisation. It was evident, that considerable interconversion of components and subunits took place suggesting an equilibrium mechanism. A relationship between component A and the α_2- subunit, between component B and the α_1- subunit, between component C and the β_{11}- subunit and between component D and the β_{12}- subunit was suggested. Furthermore, in the case of both α_2 and component A, considerable amounts of material eluting in the α_1 position were

recovered. As the evidence from both sedimentation and gel-filtration (see below) does not indicate excessive thermal degradation of either α_2 or component A resulting in low molecular weight products, the character of these degraded fractions would appear to resemble the α_1- subunit.

Such an equilibrium between subunits and components would also explain the nature of the melting curves obtained for both subunits α_1 and α_2, as well as for components A and B. At the same time, the similar mutarotation characteristics of the subunits and components indicating high helical contents, may also be explained by such a mechanism.

The evidence from gel-filtration was found to support the above conclusions. In the case of the α_1- and α_2- subunits, monodisperse molecular weights were indicated, while both components A and B yielded composite peaks indicating the presence of two high molecular weight moeities. No evidence for the excessive thermal degradation of subunits or components resulting in low molecular weight products was obtained, although gel-filtration did indicate the relative instability of the α_2 and A fractions. The low mutarotation characteristics of the α_2- subunit would also suggest such a relative instability.

Bearing in mind the caution necessary in comparing denatured collagen subunits from different sources, and separated by methods

differing in detail, the components A, B, C and D may be related to the chromatographic isolation of similar components reported in earlier findings. The Y-component reported by Schleyer (1962), which was not identified by sedimentation, has elution properties on CM-cellulose similar to component A. Tristram et al. (1965) relate their component 3 with the Y-component of Schleyer, and showed the thermal degradation of both their components 3 and 4 at temperatures above 37.5°. Component 4 was eluted with 0.1N sodium hydroxide, as was the X-component reported by Kulonen et al. (1962). Such strong elution conditions would be likely to elute components B, C and D as a single peak, making any correlation difficult. Francois et al. (1965) using gel-filtration on Sephadex G200 and gel-electrophoresis conclude that their components x and y have a molecular weight greater than β, but less than that of γ. Such evidence is not sufficient to establish exact molecular weights, and so these components may correspond to the present A and B components, especially in view of the wide range of molecular weights reported for the α-, β- and γ- subunits of collagen (see summary by Harding, 1964).

The elution character of the intramolecular β_{22}- subunit reported by Bornstein et al. (1964) compares closely to that of the present component A. It may be noted that Piez et al. (1963) use the exhaustive phosphate precipitation method (Gross, 1958) in

preparing acid-soluble collagen, and do not report the presence of components other than the α- , β - and γ - subunits. On the other hand Bornstein et al. (1964) as well as other workers (Schleyer, 1962 ; Hollmèn and Kulonen, 1964a and b ; Cooper and Davidson, 1965 ; Francois and Glimcher, 1965 ; Tristram et al. 1965 ; Veis and Anesey, 1965), do not use this phosphate method and all show the presence of additional components. The alkaline de-polymerisation of collagen by phosphate reprecipitation would explain such findings if these additional components are polymeric in nature.

It may be argued that the step-wise gradient system of eluting these components as also carried out by several workers may introduce elution artifacts. Cooper and Davidson (1966b), using a continuous phosphate buffer gradient, however, showed the presence of at least six components in thermally denatured acid-soluble and neutral-salt-soluble collagen. Furthermore, the elution pattern using the phosphate buffer gradient bears a striking resemblance to that obtained by method (b).

The possibility of subunit aggregation must also be considered, although the available evidence is against such a mechanism taking place at 40°. Boedtker and Doty (1954) and Engel (1962) show that the aggregation of gelatin is only temperature dependent below the equilibrium melting temperature. Furthermore, the well defined resolution of these

components would appear to question any aggregation mechanism.

While the evidence presented suggests that the major components A and B have molecular weights similar to the β- subunit, and that an equilibrium relationship between subunits and components takes place at 40°, a tentative intermolecular relationship is inferred. Such a relationship is also suggested from the chromatography of the original preparations, where it was noted that the recoveries of subunits and components resulted in a rather consistent ratio, viz. $\alpha_1/B = \alpha_2/A = \beta_{11} + \beta_{12}/C+D$.

If the present A and B components may be related to the components 3 and 4 of Tristram et al. (1965) who show that these components, originally comprising the whole of the preparation, progressively break down to the α- and β - subunits with slight increases in temperature above the denaturation temperature of the parent material, then the possibility that acid-soluble collagen is a tropocollagen dimer must be considered. Backerman and Hersh (1964), using evidence from sedimentation, report the presence of only β-subunits in an acid-soluble extract from new born human skin and conclude that "the molecular weight of the undenatured soluble collagen monomer must be a whole-number multiple of the molecular weight of the β - chain." This conclusion would only be justified if the parent material was in fact dimeric.

While numerous values for the intrinsic viscosity of collagen appear in the literature (see summary by Kahn and Whitnauer, 1966), the absence of values extrapolated to zero rates of shear make any comparison difficult. Davison and Drake (1966) quote a value of 10 dl/g for pronase treated monomeric tropocollagen at zero rate of shear, and attribute their higher values (17-20 dl/g) for untreated preparations as being due to the presence of linear polymers. Kahn and Whitnauer (1966) quote a value of 27 dl/g for acid-soluble collagen at low rates of shear which would support the theoretical supposition that the collagen dimer is terminally linked with a 10% overlap (Hodge and Petruska, 1963), and would display an intrinsic viscosity of approximately 30 dl/g (Mehl et al. 1940). This evidence would strongly suggest that acid-soluble collagen is dimeric.

If the suggestion regarding the dimeric nature of acid-soluble collagen is correct, then the components A, B, C and D may be related to the terminally linked dimeric subunits α_2-α_2 , α_1- α_1 , β_{11}-β_{11} and β_{12}-β_{12} respectively. Such an intermolecular relationship would appear to explain many of the present experimental findings.

From the argument presented above, it is evident that when using data from sedimentation studies only, extreme caution is necessary in the interpretation of results. Considerable supporting evidence has been presented for the β - like sedimentation characteristics of components A and B, the presence of which would explain the wide range

of α/β ratios reported in the literature. Such a finding would also explain the nature of the β_{22}- subunit reported by Bornstein et al. (1964). While the evidence for relating the minor components C and D as being terminally linked β - subunits is at present speculative, it is of interest to note that the molecular weights of these components would be in the region of 350,000 if the value of 260,000 for tropocollagen is accepted (Davison and Drake, 1966). Drake et al. (1966) report the presence of a minor component X which they conclude "must be a compact form of γ - tropocollagen, or a structure containing 4 or more α chains arising from an intermolecular bonding between two or more collagen molecules." A correlation with the components C ($\beta_{11} - \beta_{11}$) and D ($\beta_{12} - \beta_{12}$) would appear to be likely. It is also of interest to note that Drake et al. (1966) report that the β - content of soluble collagen is reduced from 65% to 0% by pronase treatment, but that the same treatment only reduces the β content of insoluble collagen from 56% to 10%. Furthermore, their β - component from pronase treated tropocollagen was found to be devoid of β_{11} when examined chromatographically on CM-cellulose. Such an interpretation of sedimentation characteristics may well explain in part at least the widely differing molecular weights of tropocollagen reported in the literature.

While the above conclusions would suggest that acid-soluble collagen is in fact dimeric tropocollagen, the possible monomeric

nature of neutral-salt-soluble collagen must be considered. Evidence for such an intermolecular relationship is presented in Chapter VI.

CHAPTER IV.

THE EFFECT OF ULTRAVIOLET IRRADIATION ON ACID-SOLUBLE COLLAGEN.

Introduction.

The formation of collagen fibrils is thought to be due to the intermolecular interaction of the telopeptides protruding from the rigid parent molecule (Boedtker and Doty, 1956 ; Hodge and Schmitt, 1958 ; Schmitt et al. 1964). These telopeptides are rich in both tyrosine and phenylalamine (Schmitt et al. 1964 ; Hodge et al. 1960 ; Rubin et al. 1963), and play a vital role in such fibril formation. Since tyrosine and phenylalanine are both photosensitive to ultraviolet irradiation (McLaren, 1949 ; Luse and McLaren, 1963 ; Setlow, 1957 ; McLaren and Luse, 1961), a study of the photochemistry of collagen may well shed light on the role of these aromatic amino acids in fibril formation.

The effect of ultraviolet irradiation on collagen fibres or skin would indicate that both cross-linking (Bottoms and Shuster, 1963), as well as peptide bond fission (Ramanathan, 1962 ; Cooper and Davidson, 1965) may result.

In the case of soluble collagens, no evidence for cross-linking has been presented to date, although Fujimori (1965, 1966) has shown that photopolymerisation may take place. The present study is confined

Ultraviolet Irradiation.

Collagen solutions were prepared in either 0.15 N- acetic acid,

to the effects of ultraviolet irradiation on the conformational changes induced in cooled solutions of acid-soluble collagen. The effects may be compared with a similar study carried out on neutral-salt-soluble collagen (Cooper and Davidson, 1966).

The effect of ultraviolet irradiation on acid-soluble-collagen was undertaken in order to study the stability of such material, and the possibility of using ultraviolet irradiation in degradation studies whereby controlled breakdown may yield information on the molecular structure of the collagen molecule. At the same time, such a study would afford a comparison between neutral-salt-soluble and acid-soluble collagen preparations.

Materials and Methods.

The methods used in the present study have been outlined in more detail in Chapters II and III, but where modifications to these methods have been made, the necessary details are given below.

Acid-Soluble Collagen.

Preparation No. 1 : Acid-soluble collagen was prepared from the skin of a four-week old bull-calf by the method of Piez et al. (1963). Phosphate reprecipitation (Gross, 1958) was carried out once.

Preparation No. 2 : Acid-soluble collagen was prepared from the skin of a four-week premature heifer-calf as in preparation No. 1.

Preparation No. 3 :- Acid-soluble collagen was prepared from the skin of a one-week premature bull-calf by the method of Steven and Tristram (1962). Phosphate reprecipitation (Gross, 1958) was not carried out.

In all these preparations neutral-salt-soluble collagen was extracted from the skin prior to preparing the acid-soluble collagen. Extraction details are presented in Chapter II.

Ultraviolet Irradiation.

Collagen solutions were prepared in either 0.15 N- acetic acid, sodium acetate buffer (pH 4.8, I = 0.06) or in 0.15M- potassium acetate (pH 4.8) and irradiated in a cold room (0-4°) with a Hanovia UVS 220A lamp operated by a regulated power supply. The solutions were placed in fused quartz tubes at a distance of 46 cm from the lamp unless otherwise specified. The lamp was rated to give an ultraviolet intensity at 46 cm of 1680-2160 $\mu W/cm^2$ of radiation below 4000Å.

Column Chromatography.

The method was similar to Method (b) described in detail in Chapter III. Collagen preparations were irradiated in sodium acetate buffer (pH 4.8 , I = 0.06). At irradiation times in excess of 2 hr, gelling of preparations resulted. On subsequent heating at 45°, small

insoluble residues remained which were filtered off using a small plug of cotton wool. The relative quantity of insoluble residue resulting from the irradiation was dependant upon the irradiation dose offered.

Gel-Filtration on Sephadex G100.

The method was similar to method (b) outlined in Chapter III save for the eluting buffer and the column temperature. Collagen preparations were taken up and irradiated in sodium acetate buffer (pH 4.8 , I = 0.06) and eluted with 1M- calcium chloride while maintaining the column temperature at 30°.

Viscosity.

Viscosities of collagen solutions were measured in a Cannon-Fenske viscometer (size 50, BS 188). Solutions were clarified by centrifuging at 32,000 g at 5° for 90 min before irradiation. After irradiation, solutions were equilibrated at either 20 ± 0.05° or 15 ± 0.05° before taking readings. Viscosity measurements were made on solutions containing 0.1 - 1.3 mg/ml of protein in either 0.15N-acetic acid or 0.15M- potassium acetate (pH 4.8).

Optical Rotation.

Optical rotation was measured at 15 ± 0.05° either in a 40 cm water-jacketed tube using a Bellingham and Stanley polarimeter equipped

with a sodium lamp, or in a 10 mm water-jacketed tube using a Perkin-Elmer model 141 polarimeter, readings being made at 589, 578, 546, 436 and 365 mμ respectively. Irradiation kinetics were studied using the Bellingham and Stanley polarimeter, while mutarotation studies were carried out using the Perkin-Elmer model 141 polarimeter.

The procedure for following helix formation was to irradiate the collagen solutions for a given period at 0-4°, heat denature at 45° for 15 min and then cool for 45 sec under tap water. A test sample was then immediately transferred to the viscometer or polarimeter tube at 15°, and readings taken at regular intervals for periods up to 48 hr.

Amino Acid Analysis.

Amino acid composition was determined with a Beckman 120B amino acid analyzer after hydrolysis with 6N-HCl under reflux for 24 hr.

Ultracentrifugation.

The method was the same as that outlined in Chapter III. At irradiation times exceeding 2 hr, the formation of an insoluble gel at 40° made sedimentation studies impracticable.

Results.

Chromatography on CM- Cellulose.

Initial chromatography runs were carried out using method (a) in order to study the effect of ultraviolet irradiation on the subunit composition of acid-soluble collagen. Samples of preparation No. 2 (50 mg/25 ml 0.06 acetate buffer) were irradiated for 2, 6 and 17 hr respectively before running onto the column (Fig. 16). Table IX compares the resulting subunit recoveries with the non-irradiated parent material. From these recoveries it may be seen that the α_2 fraction is most labile after irradiation with ultraviolet light resulting in the elution of degraded material in the α_1 position. The α_2 peak using method (a) has been shown to be a composite of the α_2 subunit together with the components A, B, C and D (Chapter III).

Gel-Filtration on Sephadex G100.

The gel-filtration of non-irradiated acid-soluble collagen (preparation No. 3) after thermal denaturation at 45° resulted in the elution of one major peak and two minor peaks (Fig. 17). The major peak corresponded to the subunits and components (see Chapter III) while the minor peak eluting at 700 ml was shown to correspond to the acetate buffer used in preparing the sample. The minor peak

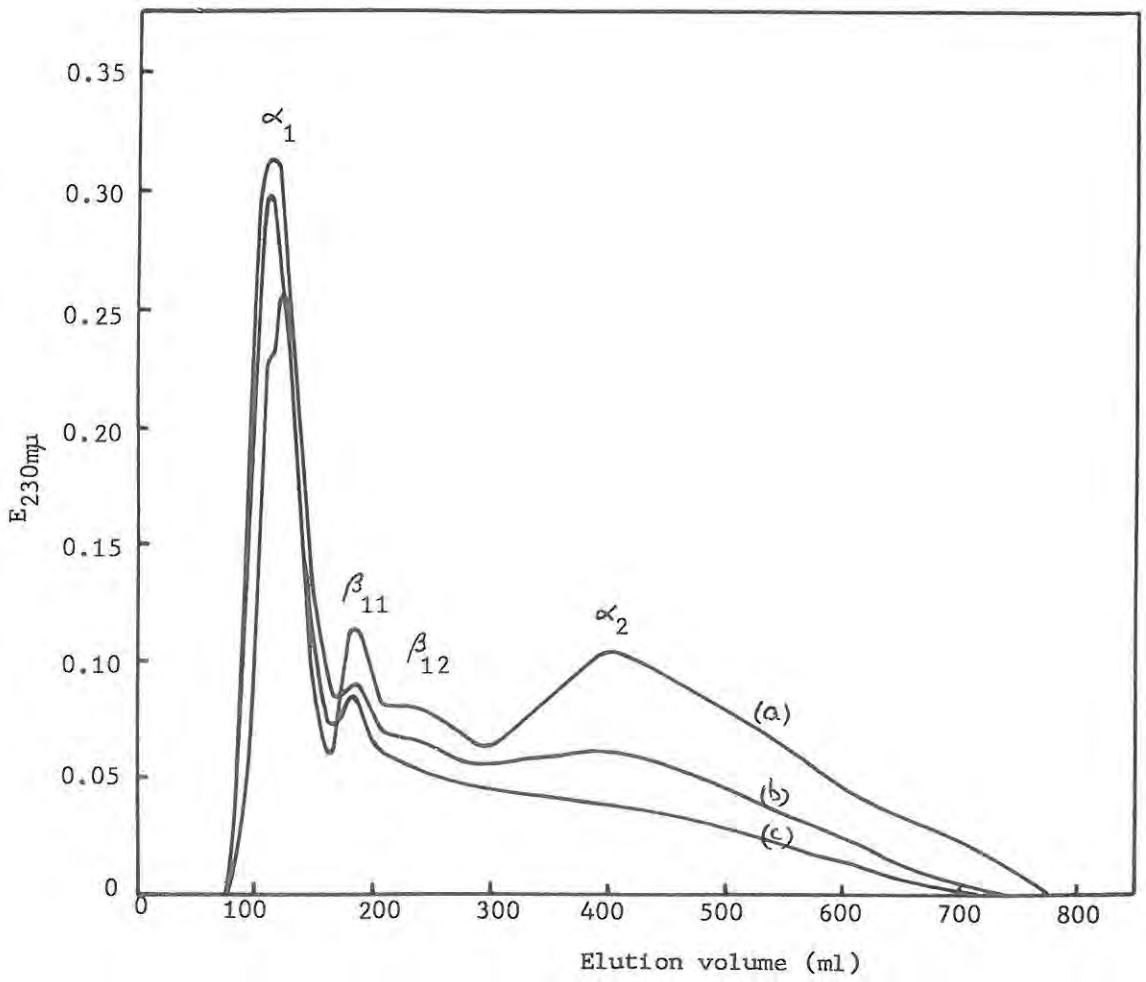

FIG. 16. Elution patterns of approximately 50 mg of irradiated acid-soluble calf-skin collagen on CM-cellulose at 40° after denaturation at 45° for 30 min. The column was eluted with a linear salt gradient using 500 ml I = 0.06 acetate buffer and 500 ml I = 0.16 acetate buffer. (a) 2 hr irradiation; (b) 6 hr irradiation; (c) 17 hr irradiation.

TABLE IX.

EFFECT OF ULTRAVIOLET IRRADIATION ON THE SUBUNIT COMPOSITION OF ACID-SOLUBLE COLLAGEN.

Irradiation Dose (hr at 0-4°)	Recovery of Soluble Protein after Irradiation	α_1	$\beta_{11}+\beta_{12}$*	α_2+
0	100%	21	18	61
2	96%	23	20	57
6	80%	37	20	43
17	70%	49	20	31

* incomplete resolution of the β_{11} and β_{12}- subunits after irradiation made on individual assessment impossible.

\+ the α_2 fraction was found to be a composite of the α_2-subunit and components A, B, C and D (Davidson and Cooper, 1967 b).

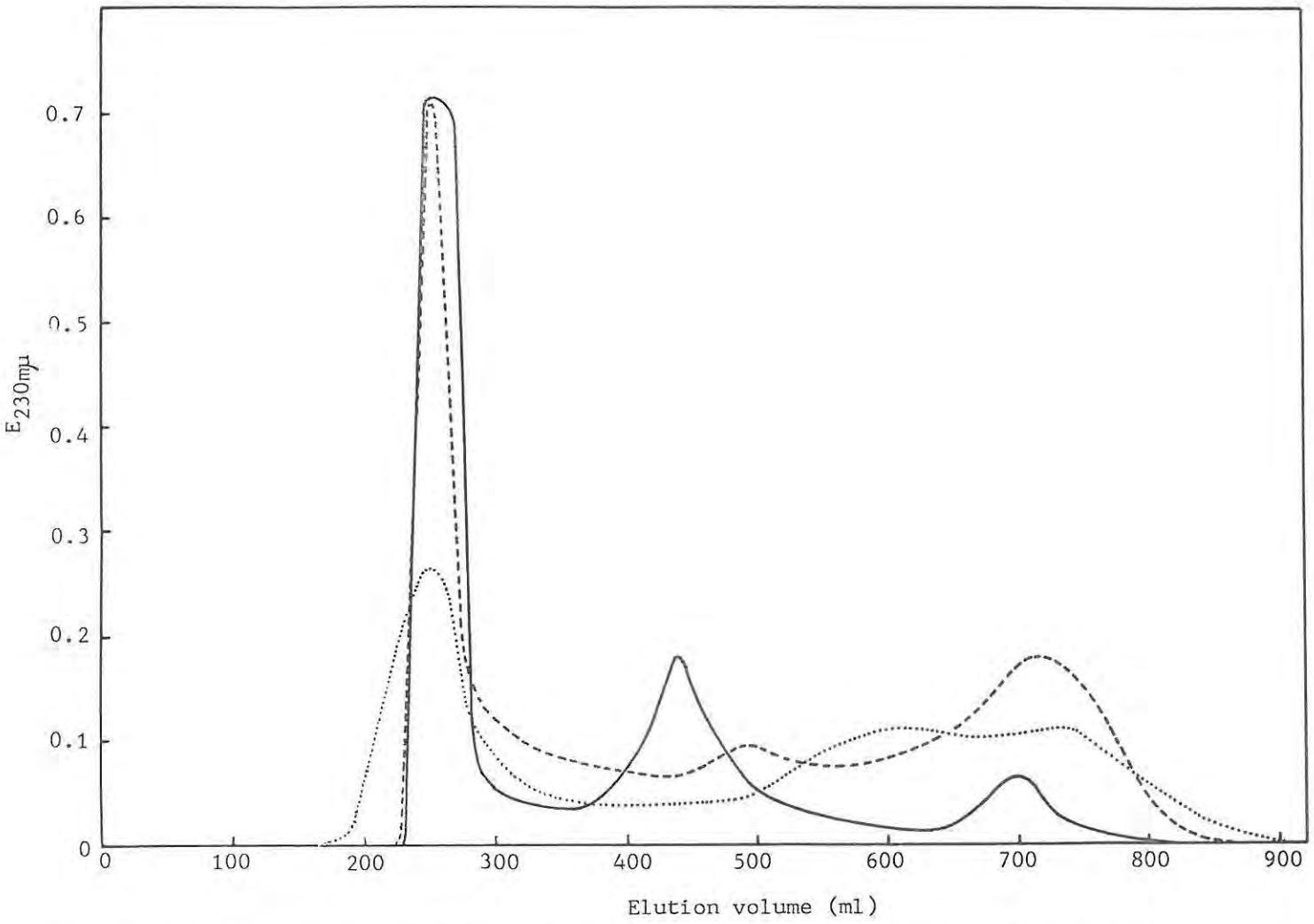

FIG. 17. Gel-filtration on Sephadex G100 at 30°of irradiated acid-soluble calf-skin collagen. 40 mg of soluble collagen in 20 ml sodium acetate buffer (pH 4.8; I = 0.06) was heated at 45° for 60 min and eluted with 1.0M-$CaCl_2$. Full line - unirradiated; broken line - 19 hr irradiation: dotted line - 38 hr irradiation.

eluting at 440 ml was initially confusing, but it was later shown that native acid-soluble collagen was eluted in this position. It was concluded that some renaturation of the thermally denatured starting material resulted under the prescribed conditions.

The gel-filtration of irradiated acid-soluble collagen (preparation No. 3) shows the decrease in the major peak and virtual elimination of the minor peak eluting at 440 ml. with the corresponding increase in the low molecular weight peak eluting at about 700 ml (Fig. 17). As the fractionation range of Sephadex G100 is approximately 4,000 - 150,000 (M_w) for globular proteins, the molecular weight of the degraded material eluting with the acetate buffer would probably be less than 4,000. In the case of both the 19 and the 38 hr irradiation, gelling resulted together with the formation of an insoluble precipitate which was filtered off before running onto the column.

Ultracentrifugation.

The ultracentrifugation of thermally denatured acid soluble collagen (preparation No. 3) resulted in the sedimentation of the α- and β - subunits with sedimentation coefficients $S_{20,w}$ 2.6 and 3.4 respectively (Chapter III). The ultracentrifugation of thermally denatured acid-soluble collagen which had been irradiated for 2 hours resulted in a similar sedimentation pattern with sedimentation coefficients $S_{20,w}$ 2.5 and 3.4 respectively, but a considerable decrease

in the β- peak and an increase in the α- peak was observed (Fig. 18).

Viscosity.

The decrease in reduced viscosity of acid-soluble collagen at various concentrations (preparation No. 1) as a function of irradiation time is shown in Fig. 19. The reaction rate plots for viscosity were obtained from the relationship between log $(d\eta/dt)$ and t, as derived from equ.(1) (von Hippel and Harrington, 1959):

$$\ln (d\eta/dt) = \ln (-k\eta_o) - kt \qquad (1)$$

where η is the reduced viscosity at time t, η_o is the total change in reduced viscosity during the reaction and k is the apparant first-order rate constant. The results (Fig. 20) show that the change in viscosity induced by irradiation is a first order reaction, with a much greater rate constant, k, than that for optical rotation (see below and Fig. 21). The rate constant of 10.5×10^{-3} min^{-1} for acid-soluble collagen at a concentration of 0.042 g of protein per 100 ml compares favourably with that reported for neutral-salt-soluble collagen at a similar concentration (Cooper and Davidson, 1965).

The rate constant k was found to be inversely related to concentration (Fig. 21). Therefore at the concentrations used in chromatography (0.3 g/100 ml) the change in viscosity due to the irradiation will be extremely slow.

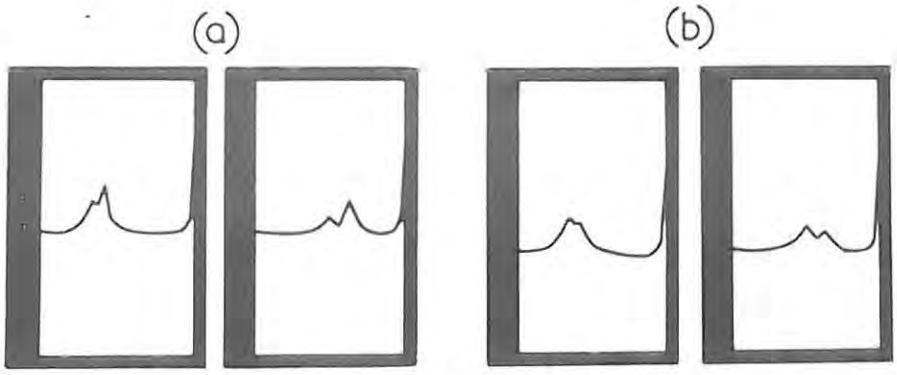

FIG. 18. Sedimentation patterns of (a) thermally denatured acid-soluble calf-skin collagen; (b) thermally denatured acid-soluble calf-skin collagen which has been irradiated for 2 hr. The photographs were taken after 80 and 120 min at 56, 100 r.p.m. and 35°. The solutions contained 0.4% ($^{w}/_{v}$) of protein in sodium formate buffer (pH 3.75 and I = 0.15). Sedimentation is from left to right.

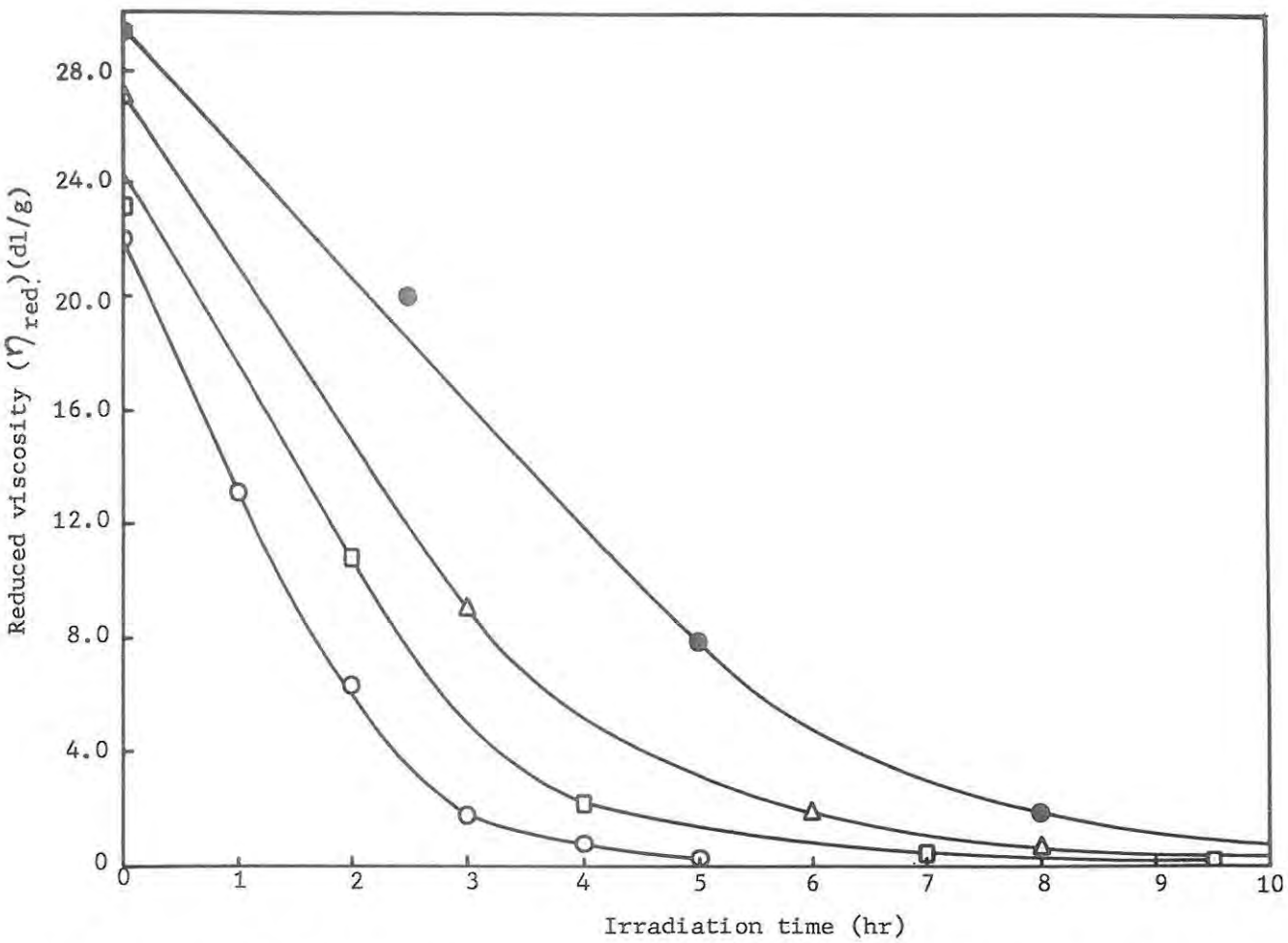

FIG. 19. Effect of ultraviolet irradiation on the reduced viscosity of acid-soluble calf-skin collagen in 0.15M-HAc. ● , 0.041% ($^{w}/_{v}$); △ , 0.033% ($^{w}/_{v}$); □ , 0.017%($^{w}/_{v}$); ○ , 0.010% ($^{w}/_{v}$).

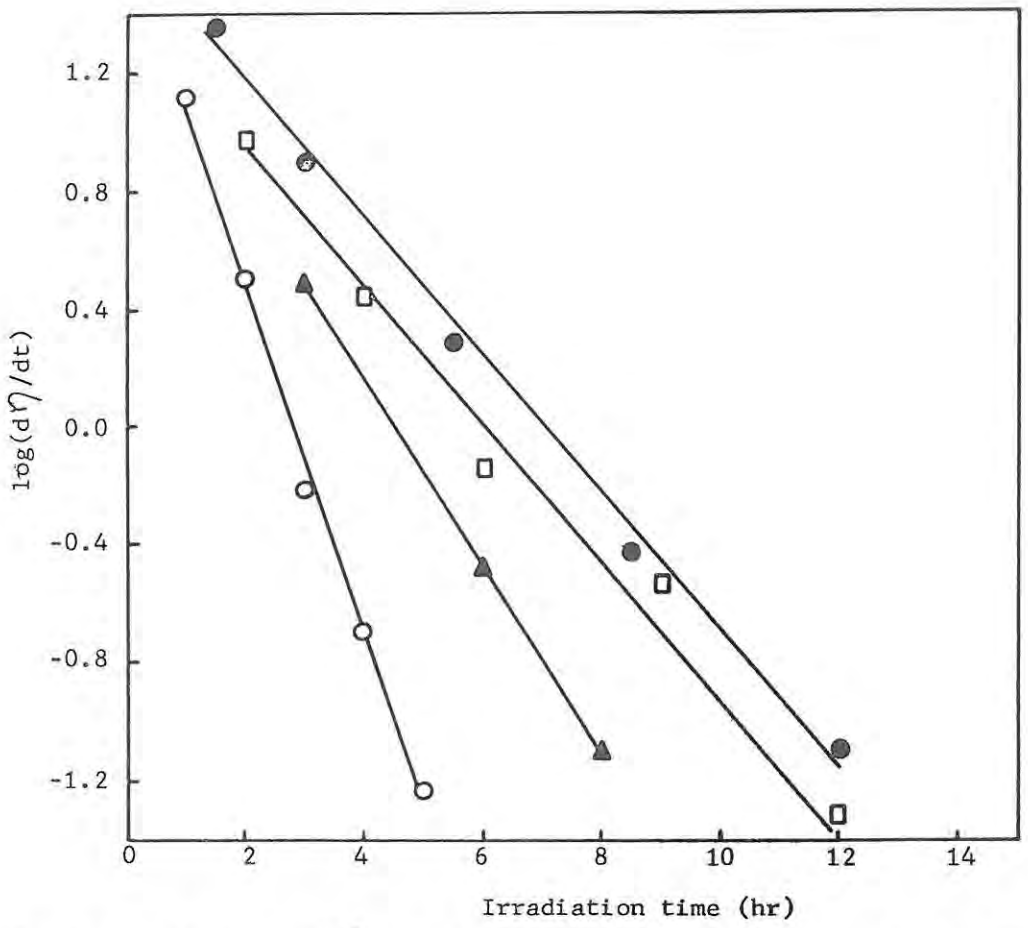

FIG. 20. Plots of log $(d\eta_{red}/dt)$ as a function of irradiation time at a distance of 46 cm at 0-4°. ● , 0.080% ($w/_v$) acid-soluble calf-skin collagen; □ , 0.042% ($w/_v$) neutral-salt-soluble calf-skin collagen; ▲ , 0.033% ($w/_v$) acid-soluble calf-skin collagen; ○ , 0.010% ($w/_v$) acid-soluble calf-skin collagen.

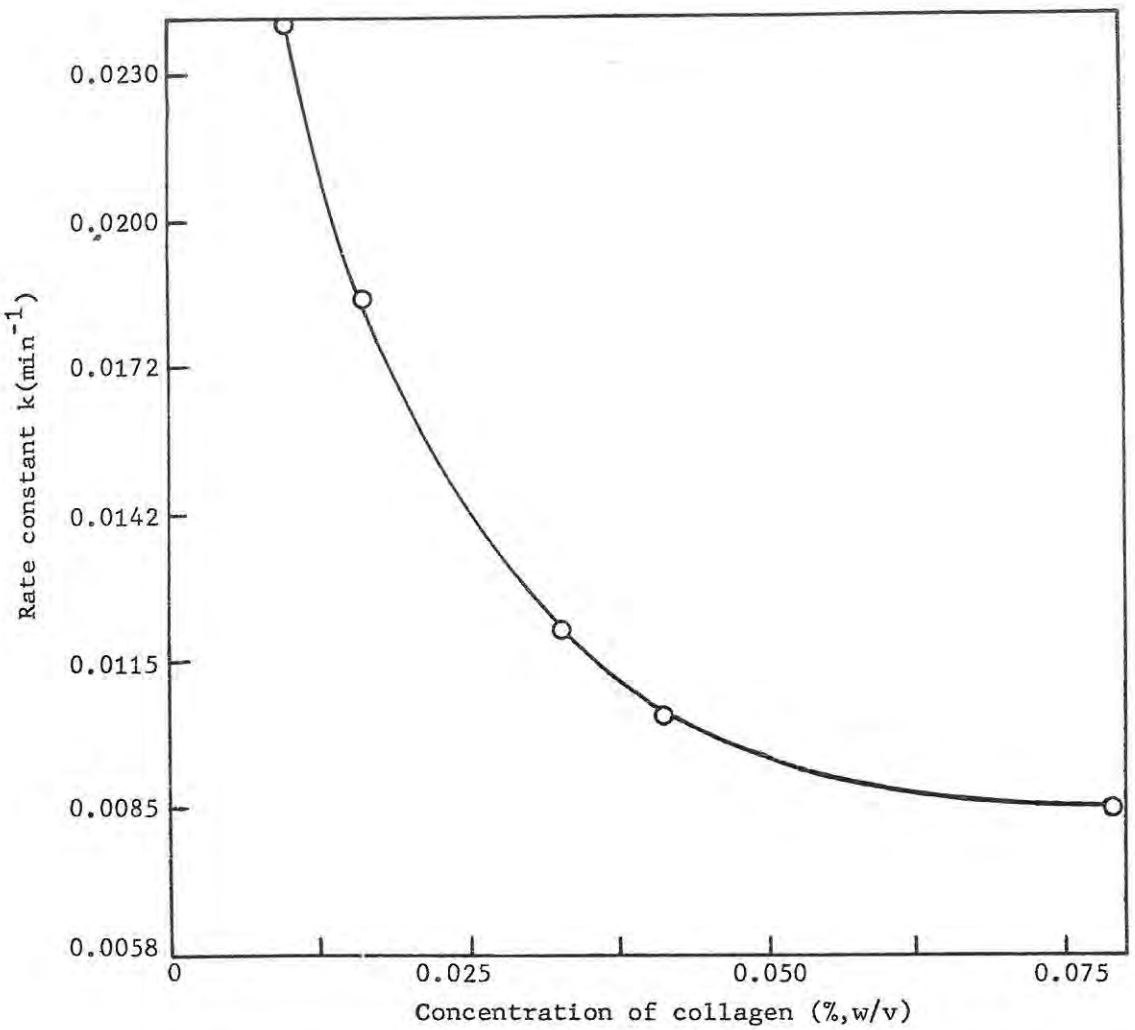

FIG. 21. First order rate constant, k, calculated from viscosity data as a function of acid-soluble calf-skin collagen concentration. Collagen was irradiated at 46 cm at 0-4°.

The fractional change in reduced viscosity as a function of irradiation time is shown in Fig. 22.

Optical Rotation.

The decrease in the specific rotation of acid-soluble collagen (preparation No. 1) as a function of irradiation time is shown in Fig. 23. The reaction rate plots for optical rotation were obtained from the relationship between log ($d[\alpha]/dt$) and t, as derived from equ. (2):

$$\ln \left(\frac{d[\alpha]}{dt} \right) = \ln (-k[\alpha_o]) - kt$$

where $[\alpha]$ is the specific optical rotation. The results (Fig. 24) show that the change in optical rotation induced by irradiation follows a first-order reaction. The rate constant of 3.7×10^{-4} min^{-1} for acid-soluble collagen at a concentration of 0.075 g protein/100 ml compares favourably with that reported for neutral-salt-soluble collagen at a similar concentration (Cooper and Davidson, 1965).

Mutarotation.

In the first series of experiments, solutions of acid-soluble collagen (1.36 mg preparation No. 2/ml) were irradiated in the presence of air for periods of up to 19 hr, denatured at 45° for 15 min and the helix formation followed by optical rotation while the solution was kept at 15° (Fig. 25). The results for a non-irradiated solution are

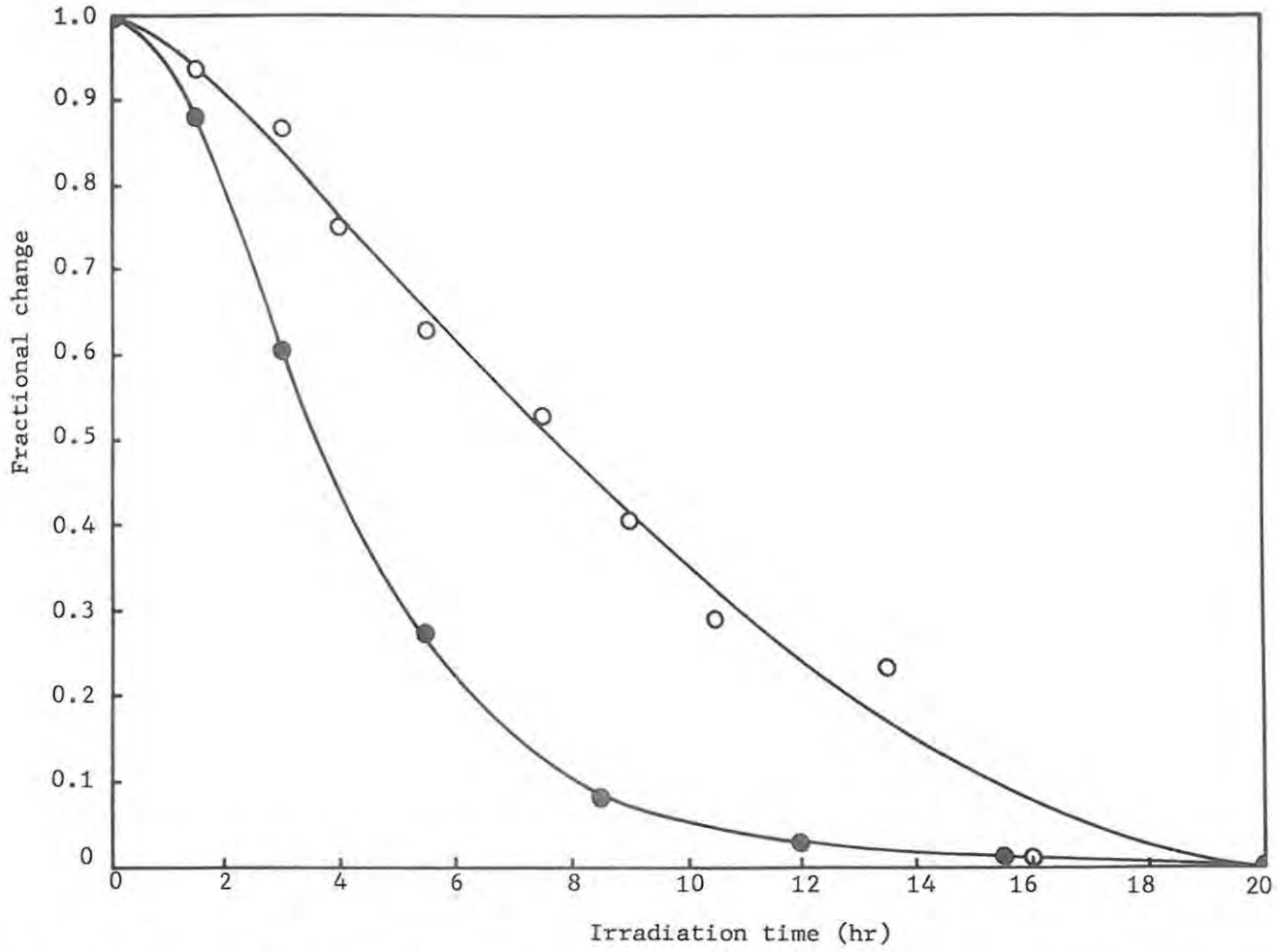

FIG. 22. Fractional change in specific rotation and reduced viscosity as a function of irradiation time at a distance of 46 cm and 0-4° for acid-soluble calf-skin collagen. ●, $(\eta_t-\eta_{20})/(\eta_0-\eta_{20})$, where η_t is the reduced viscosity at time t, and η_0 and η_{20} are reduced viscosities at zero time and after irradiation for 20 hr respectively. ○, $([\alpha]_t-[\alpha]_{20})/([\alpha]_0-[\alpha]_{20})$, where $[\alpha]_t$ is the specific rotation at time t, $[\alpha]_0$ is the specific rotation of the zero-time control, and is equal to -415°, and $[\alpha]_{20}$ is the specific rotation after irradiation for 20 hr, and is equal to -114° (0.08%, w/v).

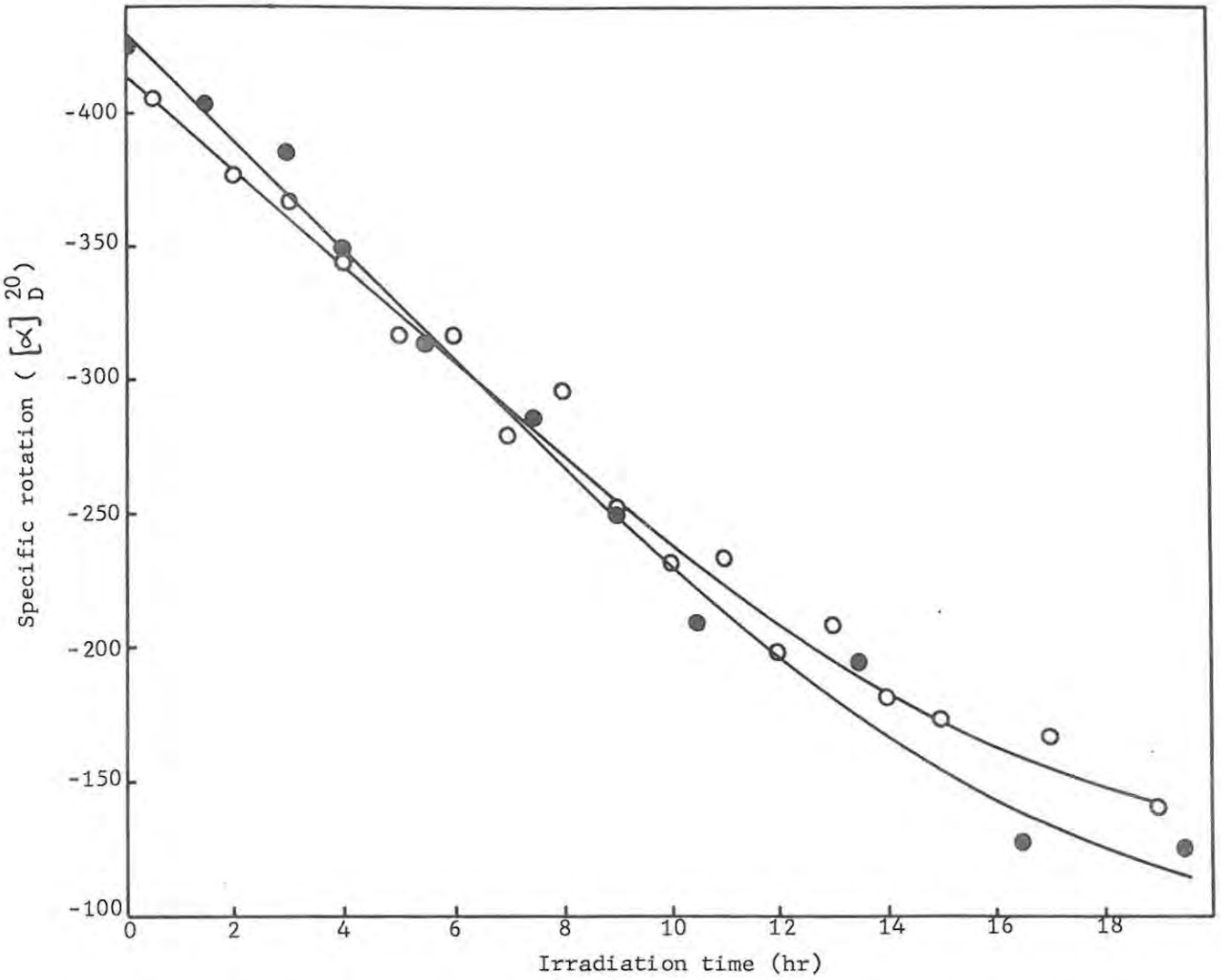

FIG. 23. Effect of ultraviolet irradiation on the specific rotation of soluble calf-skin collagen. ● , 0.080% ($^{w}/_{v}$) acid-soluble collagen; ○ , 0.074% ($^{w}/_{v}$) neutral-salt-soluble collagen. Collagen was irradiated at 46 cm at 0-4°.

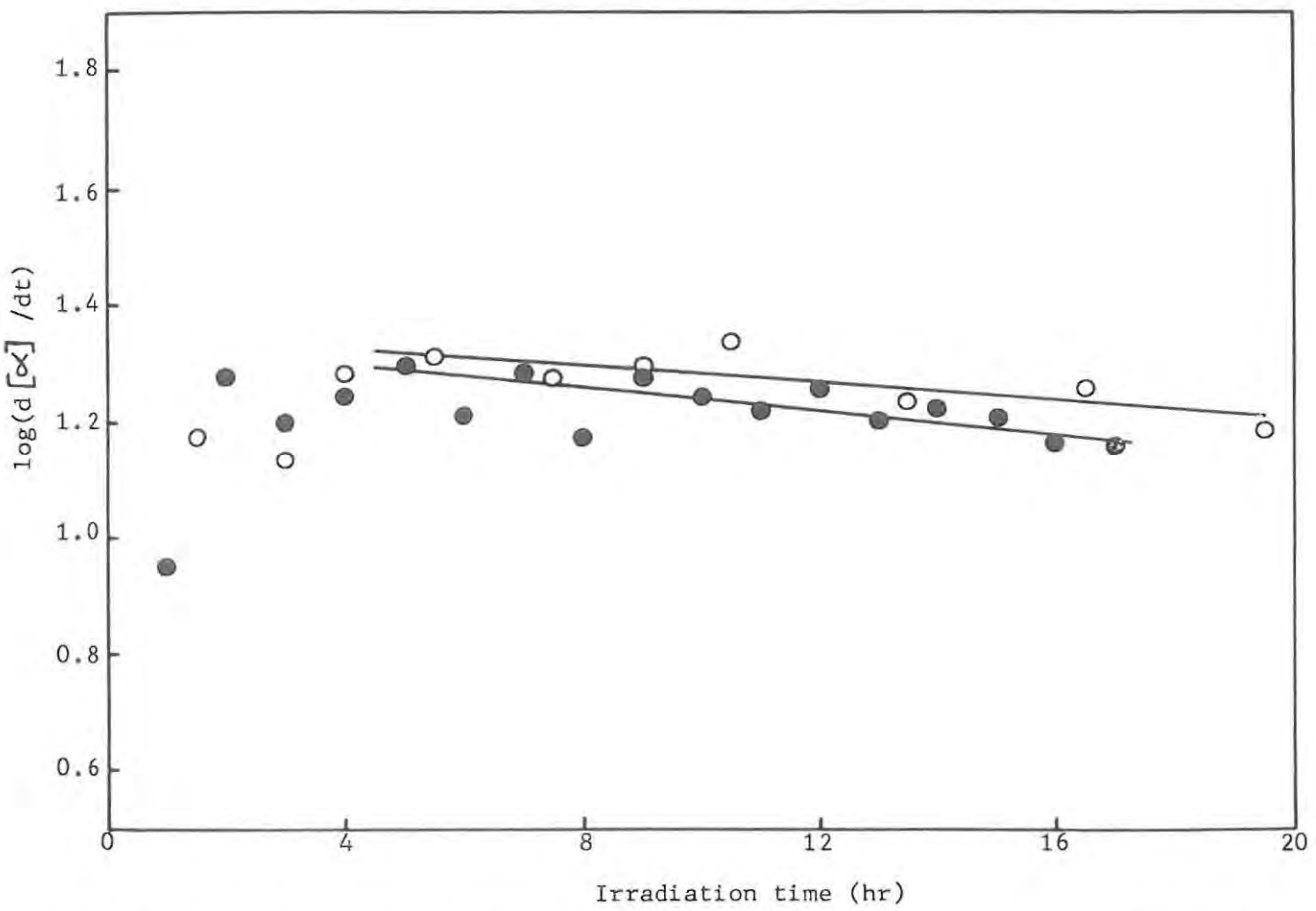

FIG. 24. Plots of $\log_{10}(d[\alpha]/dt)$ as a function of irradiation time at a distance of 46 cm at 0-4°: ●, 0.074% (w/v) neutral-salt-soluble calf-skin collagen; ○, 0.080% (w/v) acid-soluble calf-skin collagen.

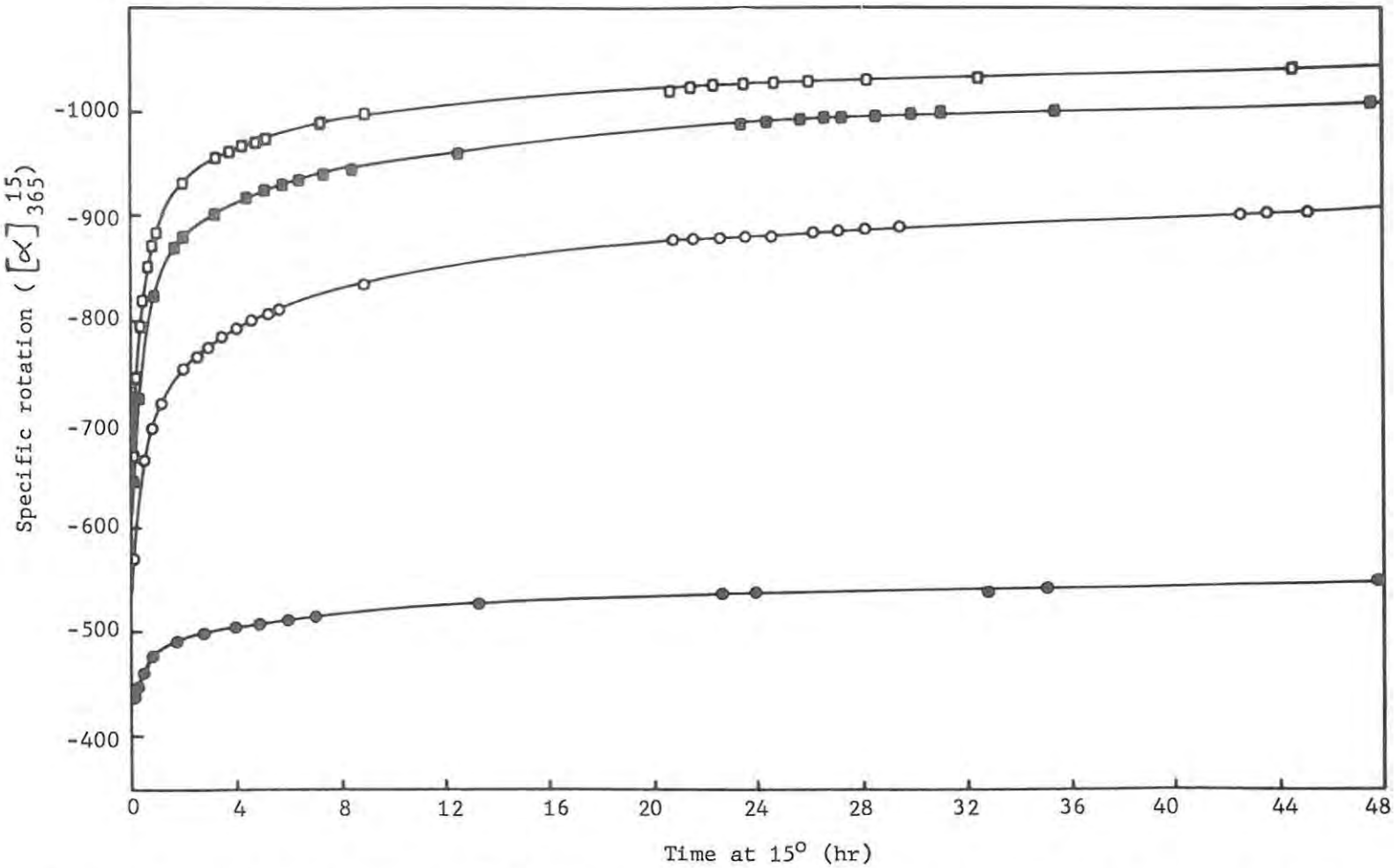

FIG. 25. Specific rotation ($[\alpha]_{365}^{15}$) as a function of time for solutions of acid-soluble collagen (1.36 mg/ml) irradiated with ultraviolet light at 0-4°, thermally denatured at 45° and then kept at 15°. □ , Not irradiated; ■ , irradiated for ½ hr; ○ , irradiated for 3 hr.; ● , irradiated for 19 hr.

included, showing a recovery of 80% of the helical content of the native collagen. This recovery for native acid-soluble collagen is considerably higher than the 59% recovery reported for neutral-salt-soluble collagen (Cooper and Davidson, 1966), but is in agreement with other reported values (50-86% ; Harrington and von Hippel, 1961).

It is evident from the irradiation data (Fig. 25 and Table X) that ultraviolet irradiation decreases the initial rate of mutarotation, i.e. $(d[\alpha]/dt)_o$, and considerably decreases the amount of helix formation in the partial re-formation on cooling of the collagen-fold or some modification of this structure (Harrington and von Hippel, 1961 ; Veis, 1964). Furthermore, at irradiation levels of about 19 hr, both the recovery of helical nature and the association of sub-unit chains (see below) virtually cease to take place. The specific rotation after 19 hr of irradiation followed by storing at 15° for 48 hr ($[\alpha]_D^{15}$ = -154°; $[\alpha]_{365}^{15}$ = -550°) was considerably lower than for the non-irradiated material, and is similar to the mean residue rotation of collagen in the denatured form ($[\alpha]_D^{15} \simeq -90°$ to -120° ; $[\alpha]_{365}^{15} \simeq -450°$), indicating almost complete loss of the poly-L-proline II - type helix.

A parallel study was also made of the change in reduced viscosity at 15° (Fig. 26 and Table X). Ultraviolet irradiation caused a decrease in the initial rate of viscosity recovery, $(d\eta_{red}/dt)_o$, and the amount

TABLE X.

RATE OF COLLAGEN-FOLD FORMATION OF ACID-SOLUBLE COLLAGEN AFTER ULTRAVIOLET IRRADIATION AND COOLING TO 15°.

Solutions were irradiated at 0-4° for the specified times, and then thermally denatured at 45° for 15 min, before being cooled to 15°.

Irradiation Time	Initial rate of mutarotation at 15° $(d[\alpha]/dt)_o$ (deg /min)		10^{-2} x Initial rate of reduced viscosity recovery at 15° $(d\eta_{red.}/dt)_o$ (dl /g.min)
	589 mμ	365 mμ	
Not irradiated	0.80	7.0	2.0
½ hr	0.59	4.3	1.2
1 hr	-	-	0.7
3 hr	0.45	3.3	0.4
19 hr	0.02	1.5	0.0

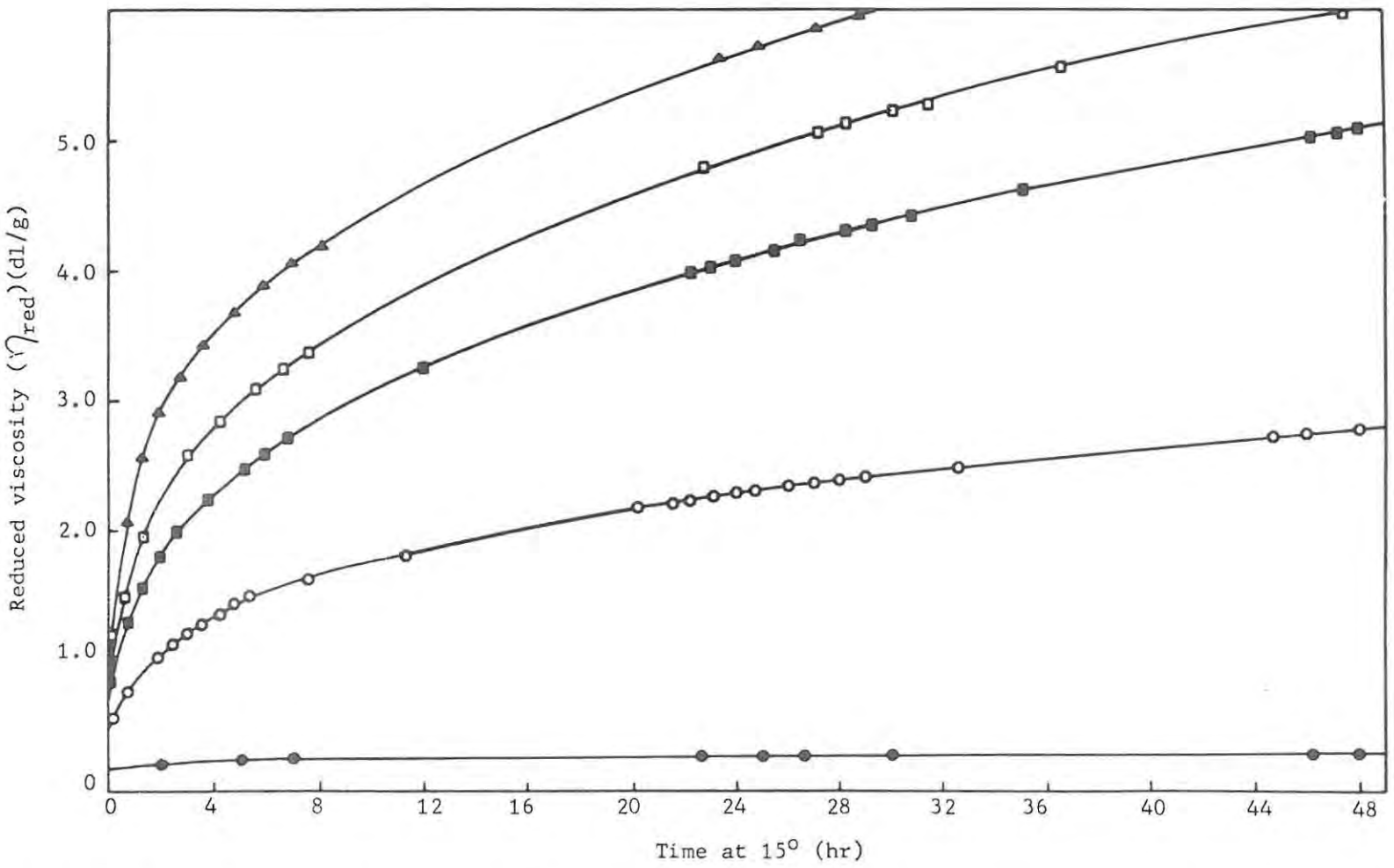

FIG. 26. Reduced viscosity (η_{red}) as a function of time for solutions of acid-soluble collagen (1.36 mg/ml) irradiated with ultraviolet light at 0-4°, thermally denatured at 45° and then kept at 15°. △, Not irradiated: □, irradiated for ½ hr.: ■, irradiated for 1 hr.; ○, irradiated for 3 hr ●, irradiated for 19 hr.

of chain association occurring parallels the regain in helical content.

Fig. 27 illustrates the relationship between reduced viscosity on cooling for 48 hr at 15° and the irradiation time. This relationship is thus a measure of aggregation or inter-chain association (Harrington and von Hippel, 1961), which is directly related to irradiation damage. The main effect of irradiation would appear to be almost complete in the first 5 hr under the prescribed conditions, indicating that the first breaks in the polypeptide chains are clearly more effective than subsequent breaks in reducing subunit chain association. The relationship between specific rotation and irradiation time is also illustrated in Fig. 27, representing the effect of irradiation damage on the helical content or intra-chain reaction (von Hippel and Wong, 1963 a, b).

Cooper and Davidson (1966) found a similar relationship on irradiating neutral-salt-soluble collagen with ultraviolet light, indicating that the first breaks in the chain are the more effective in decreasing the initial rate of mutarotation, and that the initial rate of mutarotation decreases as the chain length is diminished (von Hippel and Wong, 1963 b). It is reasonable to presume that the irradiation time is proportional to the number of breaks in the chain.

In order to determine the order of reaction for the recovery of optical rotation and reduced viscosity at 15°, with and without

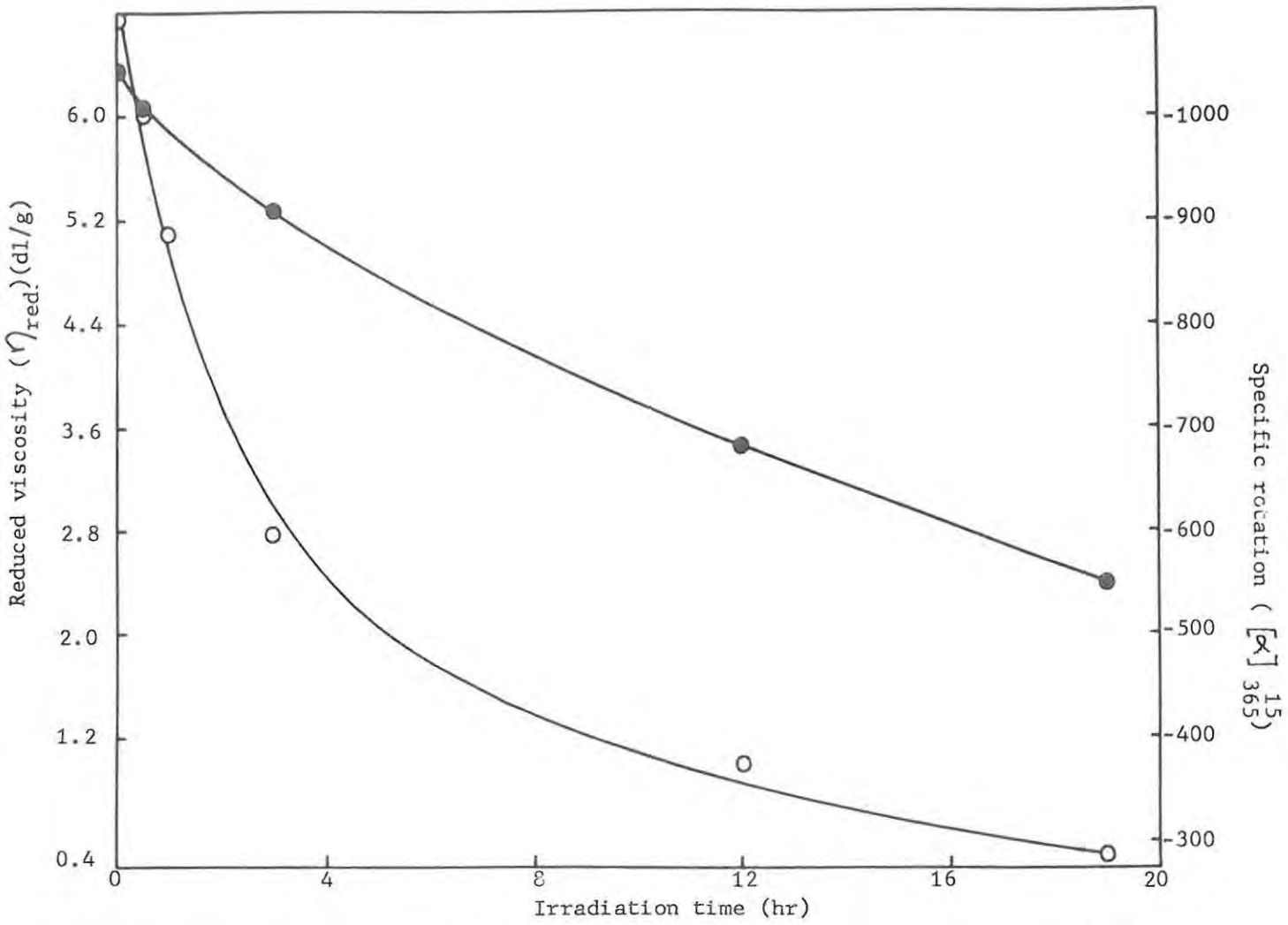

FIG. 27. Specific rotation (●) and reduced viscosity (o) as functions of irradiation time at 0-4°, for solutions of acid-soluble collagen (1.36 mg/ml) irradiated with ultraviolet light, heat denatured and then kept at 15° for 48 hr.

irradiation, log ($^{d}[\alpha]/dt$) and log ($^{d}\eta_{red}/dt$) were plotted against time. Fig. 28 illustrates a typical relationship. The non-linear relationship shows that the random coil-to-helix reaction is not a first-order reaction (Piez and Carrillo, 1964 ; Harrington and von Hippel, 1961). Similar findings were noted in the case of neutral-salt-soluble collagen (Cooper and Davidson, 1966).

Amino Acid Analysis.

The amino acid compositions of acid-soluble collagen (preparation No. 3) before and after irradiation for 19 hr are given in Table XI. The irradiated samples were first dialysed against several changes of distilled water in order to remove any degraded protein prior to analysis. These analyses show that, even though the α- and β-subunits are degraded into low molecular weight fragments by the ultraviolet irradiation, only limited changes occur in the amino acid composition. Thus the amounts of tyrosine and phenylalanine are considerably decreased.

It was also noted that preparation No. 3, both before and after irradiation, contained a small amount of 3-hydroxyproline (Piez et al. 1963) which appeared just before the methionine sulphoxide peak in the amino acid elution. After irradiation acid-soluble collagen gave a ninhydrin-positive component appearing after leucine in very small amounts (about 0.1%). Cooper and Davidson (1965) reported similar effects on irradiated neutral-salt-soluble collagen.

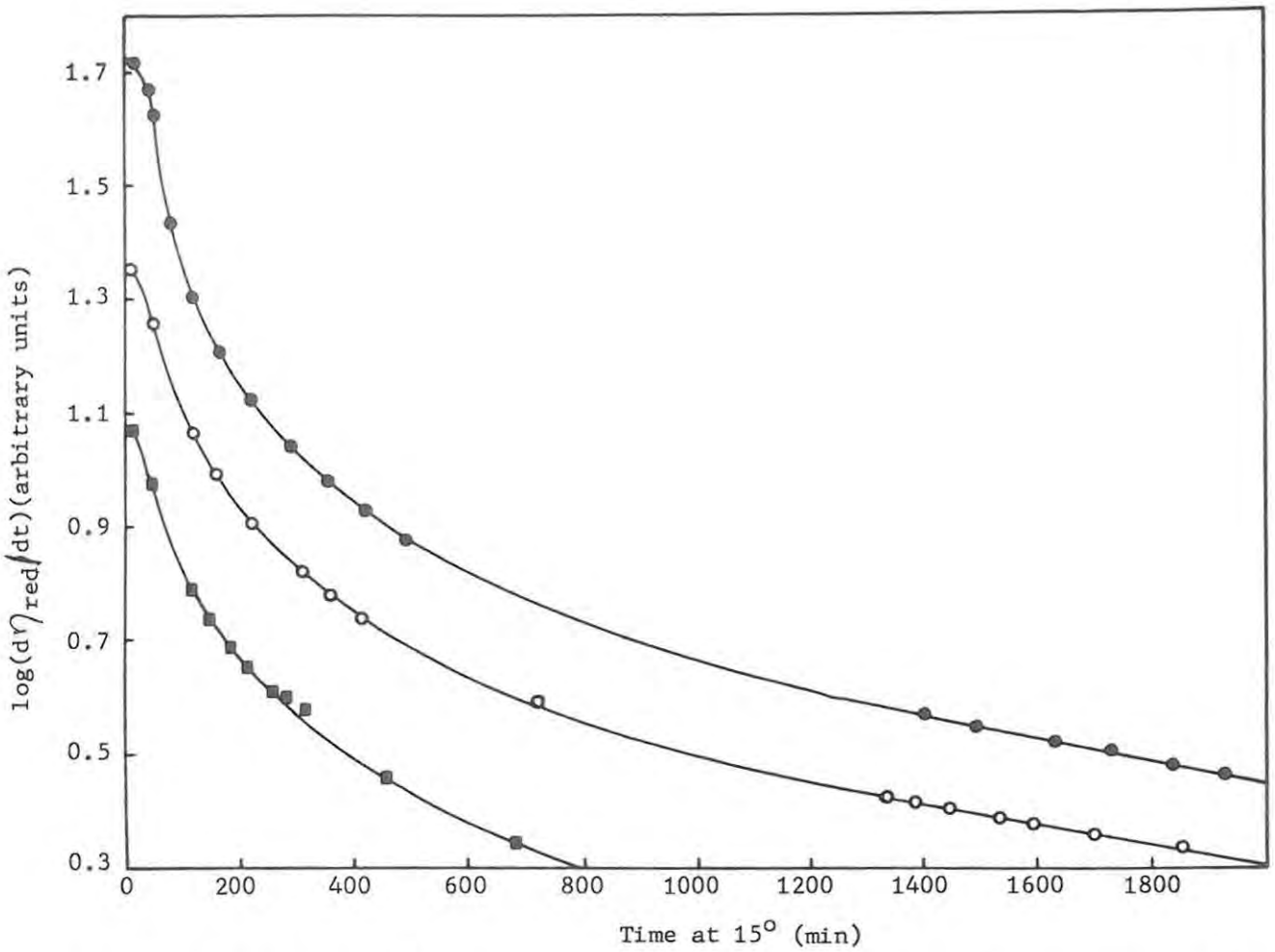

FIG. 28. Plots of log $(d\eta_{red}/dt)$ as a function of time at 15°, for solutions of acid-soluble collagen (1.36 mg./ml.) irradiated with ultraviolet light at 0-4° and thermally denatured at 45°. ●, not irradiated; ○, irradiated for 1 hr.; □, irradiated for 3 hr.

but displaying a lower axial ratio) may explain such findings. Nishihara and Doty (1958 a,b) found similar changes in these physical

Discussion.

The bulk of evidence from the ultraviolet irradiation of proteins shows that denaturation and decomposition occur (see for example Doty and Geiduschek, 1953 ; Luse and McLaren, 1963), with some evidence that ionizing radiations (Prusak and Sciarrone, 1962 ; Baily, 1963) and ultraviolet light (Kuntz, 1960, 1962 ; Bottoms and Shuster, 1963) can introduce covalent cross-links. It is known that ultraviolet irradiation does induce free radical formation (Inglis and Lennox, 1963 ; Crawshaw and Speakman, 1954) through which these cross-links could be formed.

The effect of ultraviolet irradiation on the subunit composition of acid-soluble collagen has indicated the labile nature of the α_2-subunit, as well as the dimeric components A, B, C and D (Chapter III). The resulting degradation of these subunits and components is accompanied by an increase in the relative proportion of the α_1-subunit suggesting an interconversion of subunits and components. This labile nature may be expected as the α_2- chain has been shown to be rich in both tyrosine and phenylalanine residues (Piez et al. 1963), while tyrosine is also thought to play a significant role in the intermolecular telopeptide linkage (Rubin et al. 1963). Cooper and Davidson (1965) have shown that both tyrosine and phenylalanine residues in soluble collagens are labile under the prescribed conditions.

The relative decrease in the β - subunit content indicated by ultracentrifugation would also suggest that the depolymerisation of the major components A(α_2- α_2) and B(α_1 -α_1) takes place at an early stage of the irradiation mechanism.

Although the conversion of the α_2 subunit and components A and B may initially result in α_1- like products, evidence from ultracentrifugation (Cooper and Davidson, 1966) and gel-filtration would suggest that the subsequent degradation of both α- and β- subunits into lower-molecular weight fragments takes place. The decrease in viscosity and optical rotation measurements, carried out at much lower concentration, would also suggest such degradation. It has been reported (Seifter et al. 1958 ; von Hippel and Harrington, 1960) that when collagenase reacts with icthyocol, the viscosity changes faster with time of reaction than the optical rotation. Since viscosity is a function of the axial ratio of a molecule, whereas optical rotation is related more to spatial configuration, due either to the helical natures of the individual chains or interchain alignments (Veis and Anasey, 1958 ; Drake and Veis, 1964), this is interpreted as an initial scission of the collagen molecule into polypeptides with a relatively low axial ratio, having a high helical configuration, followed by randomization of the polypeptide chains. Furthermore, if acid-soluble collagen is in fact dimeric (Chapter III) the initial depolymerisation into the monomer (having a high helical configuration

but displaying a lower axial ratio) may explain such findings. Nishihara and Doty (1958 a,b) found similar changes in these physical properties on the ultrasonic degradation of calf-skin collagen, where viscosity and sedimentation indicated that the molecule was progressively fragmented into shorter segments.

The thermal denaturation of soluble collagen has been interpreted as an all-or-nothing type of transition on the basis of viscosity and optical rotation changing at the same rate (Doty and Nishihara, 1958). The denaturation brought about by ultraviolet irradiation would appear to involve progressive degradation and decrease in chain length, with the subsequent loss in helical content. This is borne out by the fact that the optical rotation changes at a lower rate than viscosity or irradiation.

The magnitude of the first-order rate constants show that the denaturation of acid-soluble collagen brought about by ultraviolet irradiation is much slower, under the prescribed conditions, than denaturation by enzymes (von Hippel and Harrington, 1959) or thermal denaturation (Doty and Nishihara, 1958). This makes the method most useful for controlled degradation studies on the collagen structure.

Piez and Carrillo (1964) attribute the pyrrolidine ring content, the subunit content and the solvent employed as being the prime factors that control the rate and extent of mutarotation in solutions of cooled

gelatins. According to Harrington and von Hippel (1961) the re-formation of the collagen-type structure occurs in a three stage mechanism : (1) The pyrrolidine-rich portions of the peptide chain undergo initial changes that nucleate the poly-L-proline II type helix. (2) This helix structure is propagated outwards along single gelatin chains (Flory and Weaver, 1960). (3) Lateral chain association through inter-chain hydrogen bonding is made possible by the helix formation of individual chains (Veis, 1964). The first stage may be followed by amino acid analyses while the second and third stages may be monitored using optical rotation and viscometry respectively.

Since the current experiments have shown that ultraviolet irradiation effects both the rate and extent of helix formation in acid-soluble collagen, it is necessary to consider which of the above factors are affected by the irradiation. The amino acid analyses show that no significant decrease in the hydroxyproline and proline content occurs after irradiation, and therefore the initial nucleation step involving the pyrrolidene ring cannot be affected by irradiation. The amino acid analyses also show that, apart from phenylalanine and tyrosine, which are present in small amounts, no great loss of amino acids occurs on irradiation. Although tyrosine and phenylalanine are thought to play a critical role in the intermolecular interaction resulting in fibril formation (Schmitt et al. 1964), the role played by these residues in controlling the rate and extent of mutarotation

are also more effective in decreasing these initial rates than the later ones. These results are in agreement with those found by von Hippel and Wong (1963 b) for chain-fission brought about by enzymes, and confirm the conclusion that the results are independant of the method of fission.

in solutions of cooled gelatins does not appear to have been considered (Piez and Carrillo, 1964 ; Cooper and Davidson, 1966). Although the main effect of ultraviolet irradiation has been shown to be the conversion of the α- and β- subunits into smaller peptide chains, the destruction of tyrosine and phenylalanine residues resulting in considerable depolymerisation, may well be a contributory factor. The initial sharp decreases in mutarotation rates, $(d[\alpha]/dt)_o$ and $(d\eta_{red}/dt)_o$, would also suggest such a mechanism. Tyrosine and phenylalanine may thus play a critical role in the nucleation of pyrrolidine- rich portions of the peptide chain.

von Hippel and Wong (1963 b) found that the degree of helical recovery taking place on cooling decreased markedly as the molecular weight of the gelatin in solution was decreased by enzyme action. They estimate that a minimum chain length of 40-80 residues is required to generate stable elements of the collagen-type helix, and that the final helical content is independent of the method of breaking the peptide chain. The current amino acid analyses show losses of tyrosine, phenylalanine and methionine corresponding to the destruction of about 11 amino acid residues/1000 residues. This is equivalent to about 11 breaks/α- chain of molecular weight 100,000. von Hippel and Wong (1963 b) have shown that no helical content is found in cooled solutions of gelatin after enzymic reaction causes about 40 breaks/polypeptide chain of molecular weight 100,000.

As shown above, the initial rates of mutarotation and viscosity recovery are decreased by irradiation, as are the final specific rotation and reduced viscosity attained after cooling at 15° for 48 hr. The initial breaks in the polypeptide chain brought about by irradiation are also more effective in decreasing these initial rates than the later ones. These results are in agreement with those found by von Hippel and Wong (1963 b) for chain-fission brought about by enzymes, and confirm the conclusion that the results are independant of the method of fission.

Therefore, the effect of ultraviolet irradiation on the collagen-fold formation would appear to be the formation of shorter peptide chains, in the random coil form, which hinder the propagation of the helical structure starting at the pyrrolidine rings and proceeding along the single-chain random coils. This would presumably also decrease the amount of lateral chain association between single helical chains, and any single chain folding giving helices stabilized by intramolecular association (Veis, 1964).

Since the present mutarotation studies of irradiated acid-soluble collagen compare very closely with similar studies of irradiated neutral-salt-soluble collagen (Cooper and Davidson, 1966), similar intramolecular structures for both acid-soluble and neutral-salt-soluble collagens are suggested. The reason why only acid-soluble collagen forms an insoluble gel after ultraviolet irradiation is at present not apparent.

CHAPTER V.

THE EFFECT OF GAMMA IRRADIATION ON SOLUBLE COLLAGEN.

Introduction.

Some physicochemical effects of γ - irradiation on collagen have been reported. Bowes and Moss (1962) have shown a reduction in the tensile strength and shrinkage temperature of irradiated ox-hide at 5 and 50 Mrad doses. They also reported a limited destruction of acidic and basic amino acids, as well as those having a ring structure. Relatively little hydrolytic scission of peptide bonds was indicated from terminal group analysis. Ramanathan et al. (1965) have reported similar reductions in tensile strength and shrinkage temperatures for irradiated tail-tendon collagens at 10 and 30 Mrad doses, as well as modified electron micrographs, X-ray diffraction patterns and optical birefringence. Cassel (1959) irradiated tail-tendon collagen (5-220 Mrad doses) and found little damage of amino acid residues at doses less than 20 Mrad. Methionine, phenylalanine and threonine were shown to be most labile at higher irradiation doses, while alanine, glycine, hydroxyproline, proline and arginine were least affected. Strakhov and Shifrin (1964) report that at 10^4 - 10^5 rads of γ - irradiation crosslinking of collagen molecules occurs, while at irradiation levels above 10^7 rads the destructive effect predominates.

Irradiation studies on proteins have generally been concerned with effects observable in aqueous solutions in which the indirect action of free radical formation in the solvent must be taken into consideration. The greater part of the changes observed have been attributed to this latter source of potential attack on the protein. The present study is confined to the effect of γ - irradiation on the conformational changes taking place in cooled solutions of thermally denatured neutral-salt-soluble and acid-soluble collagen, after irradiation of the anhydrous protein. As in the previous study using ultraviolet irradiation (Chapter IV), the present investigation was undertaken in order to study the stability and controlled breakdown of soluble collagen using γ - irradiation.

Materials and Methods.

Preparation of Soluble Collagen.

Preparation No. 1 : Neutral-salt-soluble and acid-soluble collagen were prepared from the skin of a four-week old bull-calf by the method of Piez et al. (1963). Phosphate reprecipitation was carried out once in each case.

Preparation No. 2 : Neutral-salt-soluble and acid-soluble collagen were prepared from the skin of a four-week premature heifer-calf as in preparation No. 1.

Extraction details are presented in Chapter II.

γ - Irradiation.

Freeze-dried samples of soluble collagen were dried under vacuum over P_2O_5 for 24 hr, sealed under vacuum into glass tubes and irradiated at 22° from a 300-400 curie Cobalt-60 source. The irradiation was carried out at the Atomic Energy Board, Pelindaba. Irradiation was limited to 10 Mrad.

Column Chromatography.

The fractionation of thermally denatured irradiated collagen was carried out using the Beckman model 130 Spectrochrom Analyzer. The column and gradient used were similar to method (b) described in detail in Chapter III. The optical absorption of the effluent was monitored at 220, 230 and 278 mμ, and at two optical path lengths (2.5 mm and 10 mm). Solutions of irradiated collagen were prepared in I = 0.06 acetate buffer (2 mg/ml) and denatured at 45° for 30 min. Solutions containing 50-70 mg of denatured collagen were run onto the column and eluted at a flow rate of 100 ml/hr. The Spectrochrom also gave continuous recordings of the pH and conductivity of the column effluent, thus enabling a check to be kept on the consistency of the buffer gradient.

Ultracentrifugation.

Denatured irradiated soluble collagen was examined by sedimentation velocity at 35° as described in Chapter III.

Mutarotation at 15°.

The procedure for following helix formation of irradiated collagen in 0.15 M- potassium acetate, pH 4.8, using viscometry and polarimetry techniques has been described in Chapter IV.

Amino Acid Analysis.

The amino acid composition of irradiated collagen preparations was carried out using the Beckman 120B Analyzer, while the composition of isolated components was determined using the Beckman Unichrom Analyzer.

Two Dimensional High Voltage Electrophoresis and Chromatography.

Details of the method used have been outlined in Chapter II.

Results.

Chromatography on CM- Cellulose.

The chromatography of unirradiated thermally denatured acid-soluble collagen using the Spectrochrom method (Fig. 29) may be compared with the method discussed in Chapter IV. The present study using the Spectrochrom Analyzer has resulted in improved resolution over that obtained by the method of Piez et al. (1963), the reason being that the micro cuvette of the Spectrochrom has a much smaller volume (0.05 ml) compared with about 1 ml for most flow cells with a path length of 10 mm. Component 4 probably corresponds to the α_3-subunit reported by several authors (see for example Piez, 1964), while

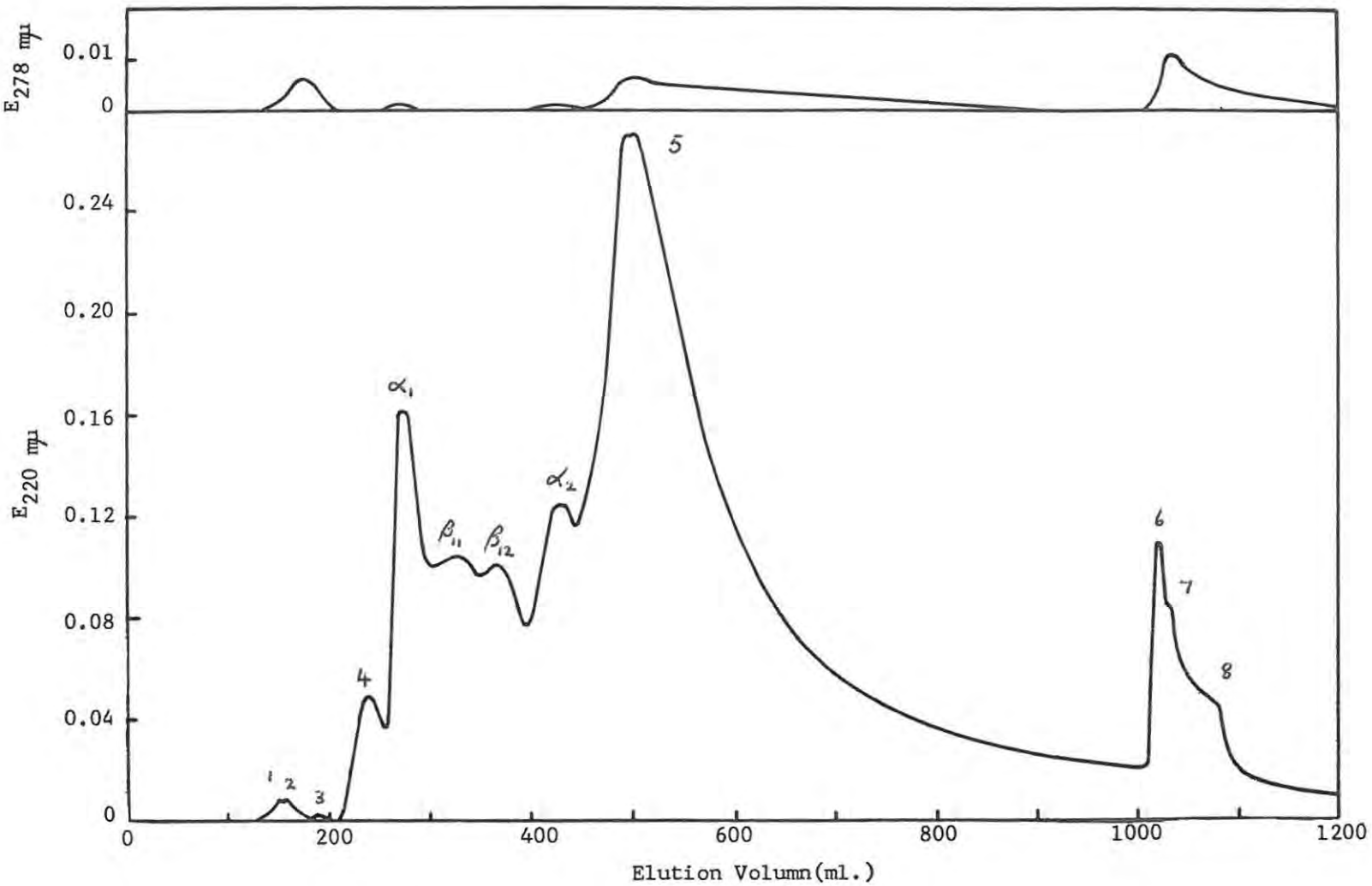

FIG. 29. Elution pattern of approximately 50 mg of acid-soluble calf-skin collagen on CM-cellulose at 40° after denaturation at 45° for 30 min. The column was eluted with a linear salt gradient using 500 ml. 1 = 0.06 acetate buffer and 500 ml. 1 = 0.16 acetate buffer followed by a change to 1 = 0.26 acetate buffer.

a correlation with the component H discussed in Chapter III is evident. The large component 5 was in some cases resolved into several peaks, and it is concluded that this component consists of the dimeric subunits α_1-α_1 , α_2-α_2 , β_{11}-β_{11} and β_{12}-β_{12} which were characterized in Chapter III (Davidson and Cooper 1967 b). The resolution of the minor components 1, 2, 3 and 6, 7, 8 has not been reported in earlier findings although components 1, 2 and 3 may be the same as the E, F and G components discussed in Chapter III. Since CM- cellulose is a weak cation exchanger, the acidic nature of components 1, 2 and 3, and the basic nature of components 6, 7 and 8, together with their relatively high absorption at 278 mμ (Fig. 29 and Table XII) due to the presence of aromatic constituents, would suggest that these components are telopeptide residues (see below). The chromatography of unirradiated thermally denatured neutral-salt-soluble collagen gave a similar elution pattern to acid-soluble collagen, except for a slightly smaller peak containing the dimeric subunits. No other differences in the four preparations studied were observed.

The chromatography of γ- irradiated neutral-salt-soluble and acid-soluble collagen at 1.3, 2.6, 5 and 10 Mrad showed the early breakdown at low levels of irradiation of the chromatographic component containing the dimeric subunits, resulting in the elution of degraded material just in front of the α_1 position (Figs. 30-33). The relatively

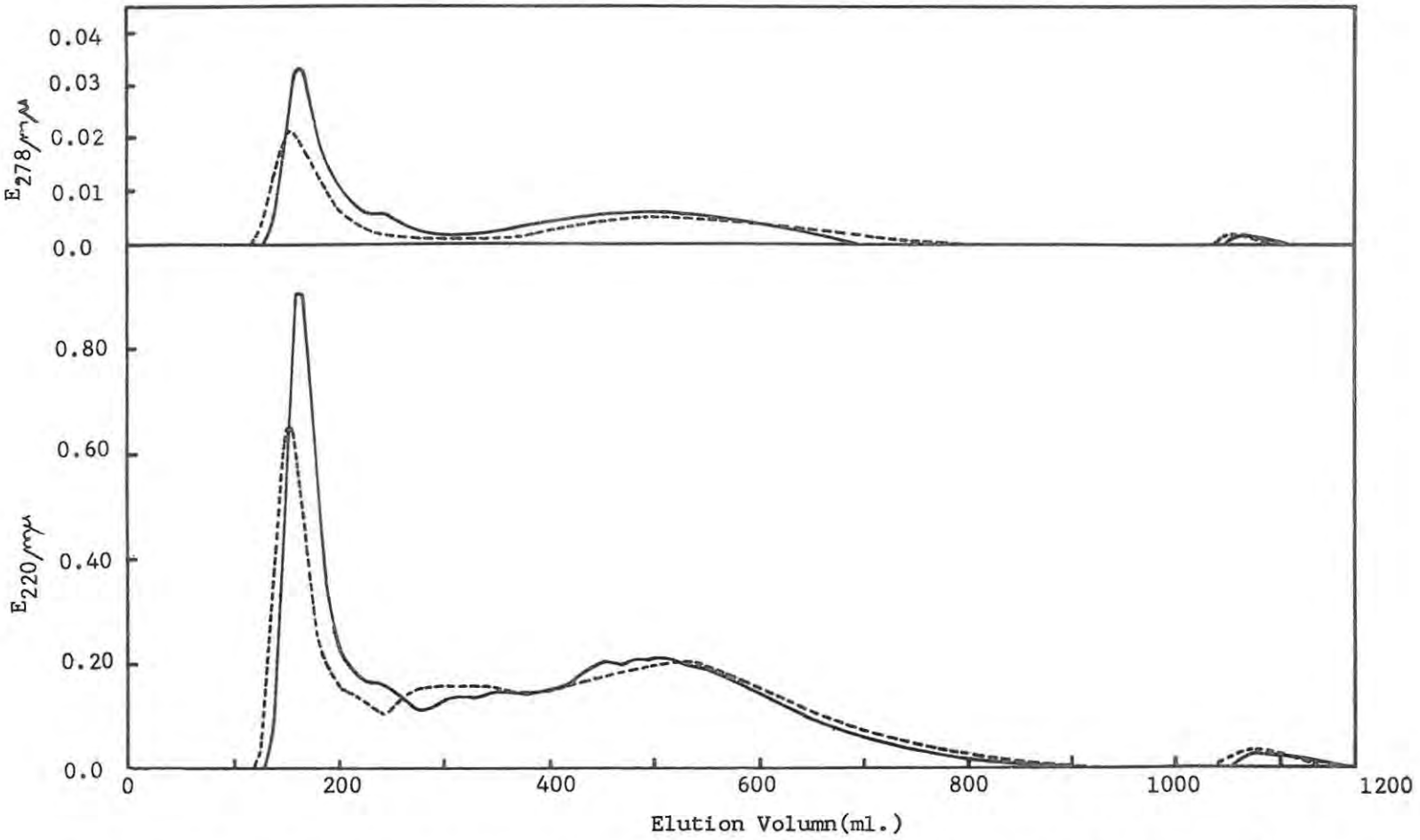

FIG. 30 Elution patterns of approximately 50mg of calf-skin collagen irradiated with 1.3 Mrad γ-irradiation: full-line-acid-soluble collagen ; dotted-line-neutral-salt-soluble collagen. The column was eluted with a linear salt gradient using 500 ml. 1 = 0.06 acetate buffer & 500 ml. 1 = 0.16 acetate buffer followed by a change to 1=0.26 acetate buffer.

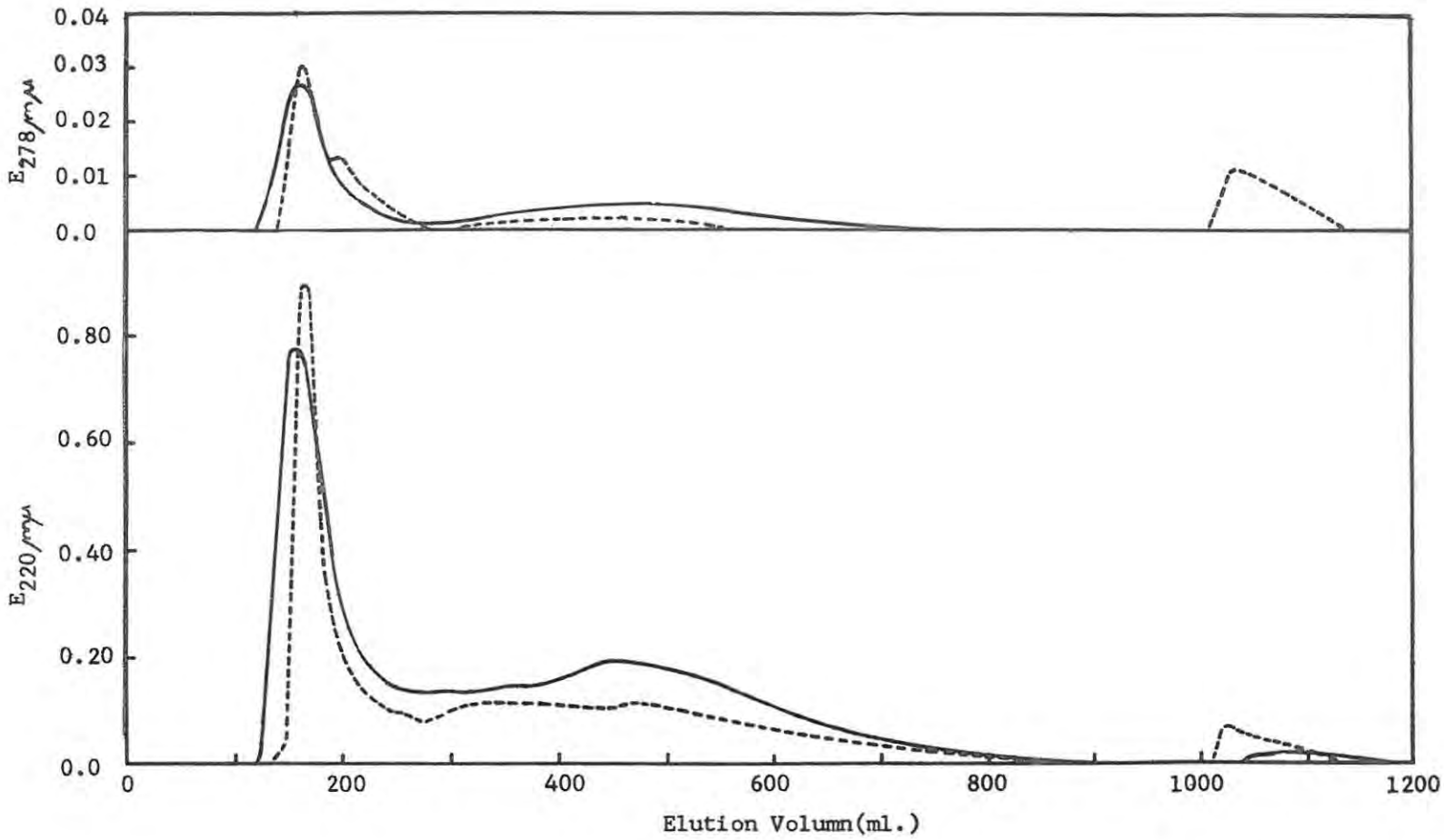

FIG.31 Elution patterns of approximately 50mg of calf-skin collagen irradiated with 2.6 Mrad γ- irradiation; full-line-acid-soluble collagen ; dotted-line-neutral-salt-soluble collagen. The column was eluted with a linear salt gradient using 500 ml. 1 = 0.06 acetate buffer & 500 ml.1 = 0.16 acetate buffer followed by a change to 1= 0.26 acetate buffer.

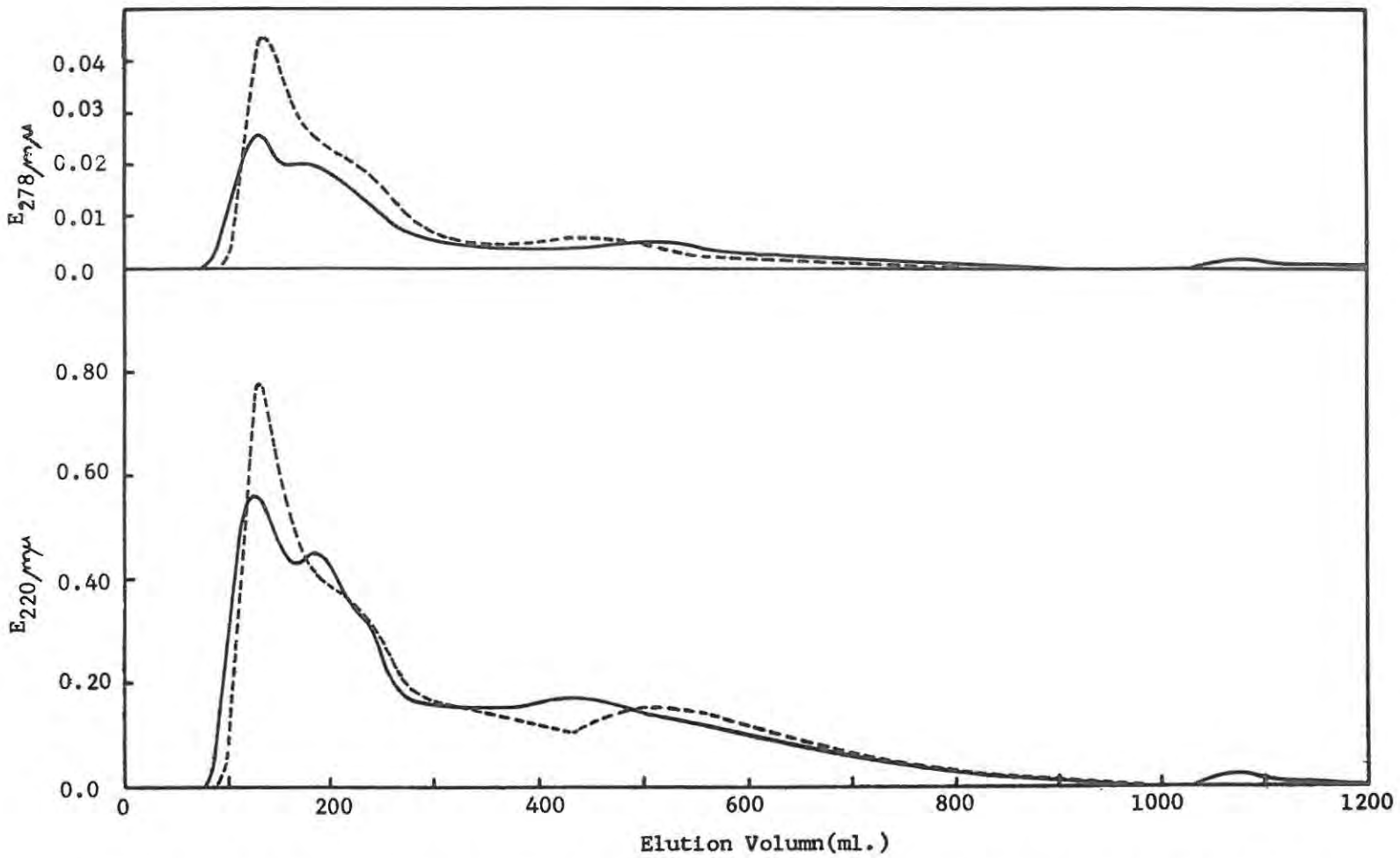

FIG. 32 Elution patterns of approximately 50mg of calf-skin collagen irradiated with 5 Mrad γ-irradiation; full-line-acid-soluble collagen ; dotted-line-neutral-salt-soluble collagen. The column was eluted with a linear salt gradient u sing 500 ml. 1 = 0.06 acetate buffer & 500 ml. 1 = 0.16 acetate buffer followed by a change to 1 = 0.26 acetate buffer.

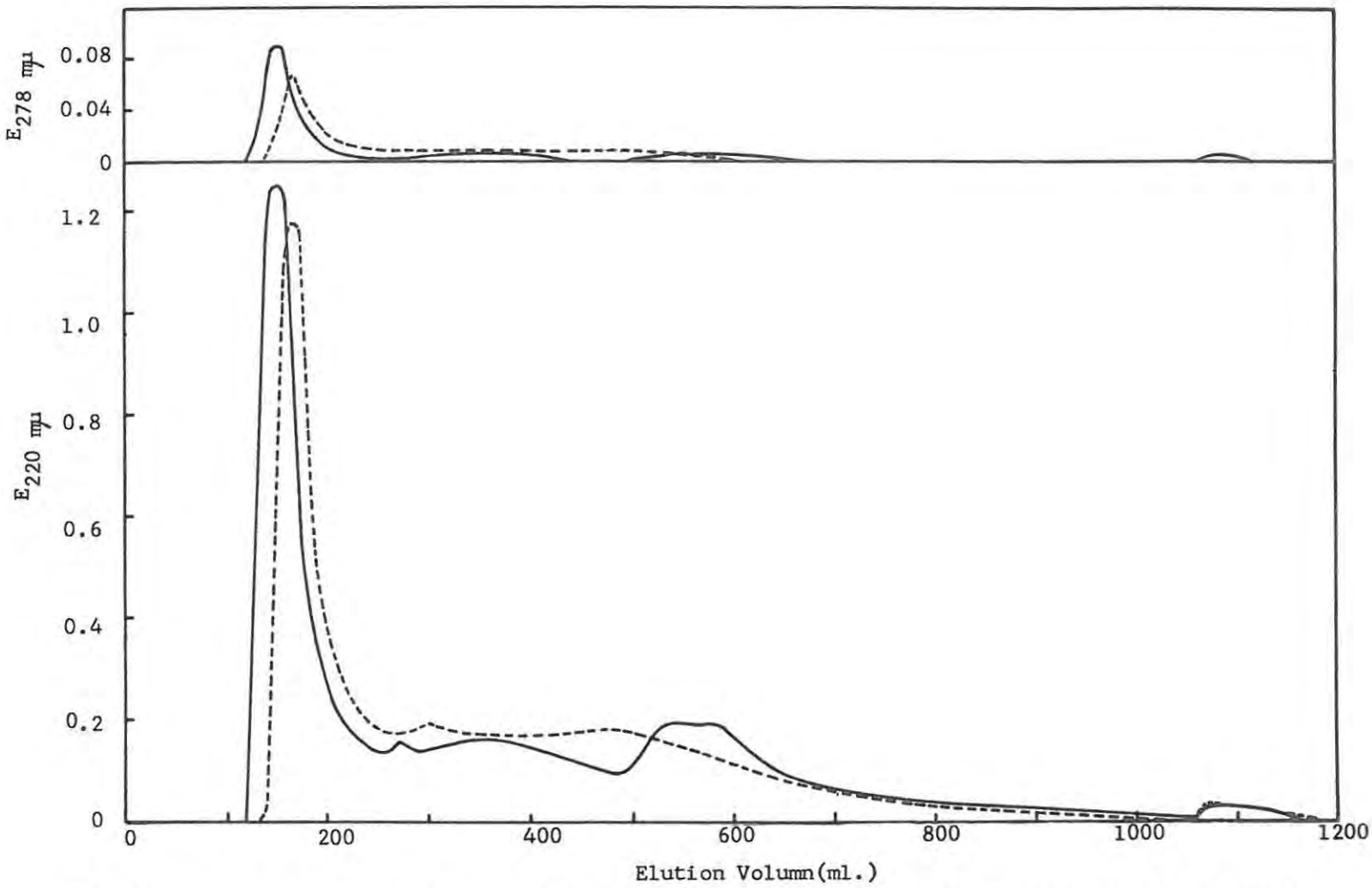

FIG.33 Elution patterns of approximately 70mg of calf-skin collagen irradiated with 10 Mrad γ-irradiation ; full-line-acid-soluble collagen ; dotted-line-neutral-salt-soluble collagen. The column was eluted with a linear salt gradient using 500 ml. 1 = 0.06 acetate buffer & 500 ml. 1= 0.16 acetate buffer followed by a change to 1= 0.26 acetate buffer.

TABLE XII.

COMPOSITION OF CHROMATOGRAPHIC COMPONENTS OF ACID-SOLUBLE CALF-SKIN COLLAGEN.

Component	% Composition. 220 mμ	278 mμ
1, 2, 3	0.5	10.8
4	2.4	0
α_1	8.2	3.3
β_{11}	6.5)	
	)	
β_{12}	5.9)	5.0
	)	
α_2	8.3)	
5	62.9	49.3
6, 7, 8	5.3	31.6

high optical absorption of this degraded material at 278 mμ was noted in all cases.

Ultracentrifugation.

The ultracentrifugation of thermally denatured neutral-salt-soluble and acid-soluble collagen both resulted in the separation of the α- and β- subunits with sedimentation coefficients $S_{20,w}$ 2.6 and 3.4, respectively (Piez et al. 1963). The ultracentrifugation of thermally denatured neutral-salt-soluble collagen which had been irradiated for 5 and 10 Mrad both resulted in a single heterodisperse peak with sedimentation coefficients $S_{20,w}$ 1.86 and 1.71 respectively. Thermally denatured acid-soluble collagen irradiated at 5 and 10 Mrad resulted in similar peaks with sedimentation coefficients $S_{20,w}$ 1.85 and 1.81 respectively (Fig. 34).

Mutarotation at 15°.

Solutions of irradiated and non-irradiated neutral-salt-soluble and acid-soluble collagen (1.3 - 1.4 mg/ml) were denatured at 45° for 15 min and the helix formation followed by optical rotation at 15°. The effect of 1.3, 2.6, 5 and 10 Mrad doses of γ- irradiation was studied in this way.

It is evident from the irradiation data (Table XIII) that both the initial rates of mutarotation as well as the actual recovery

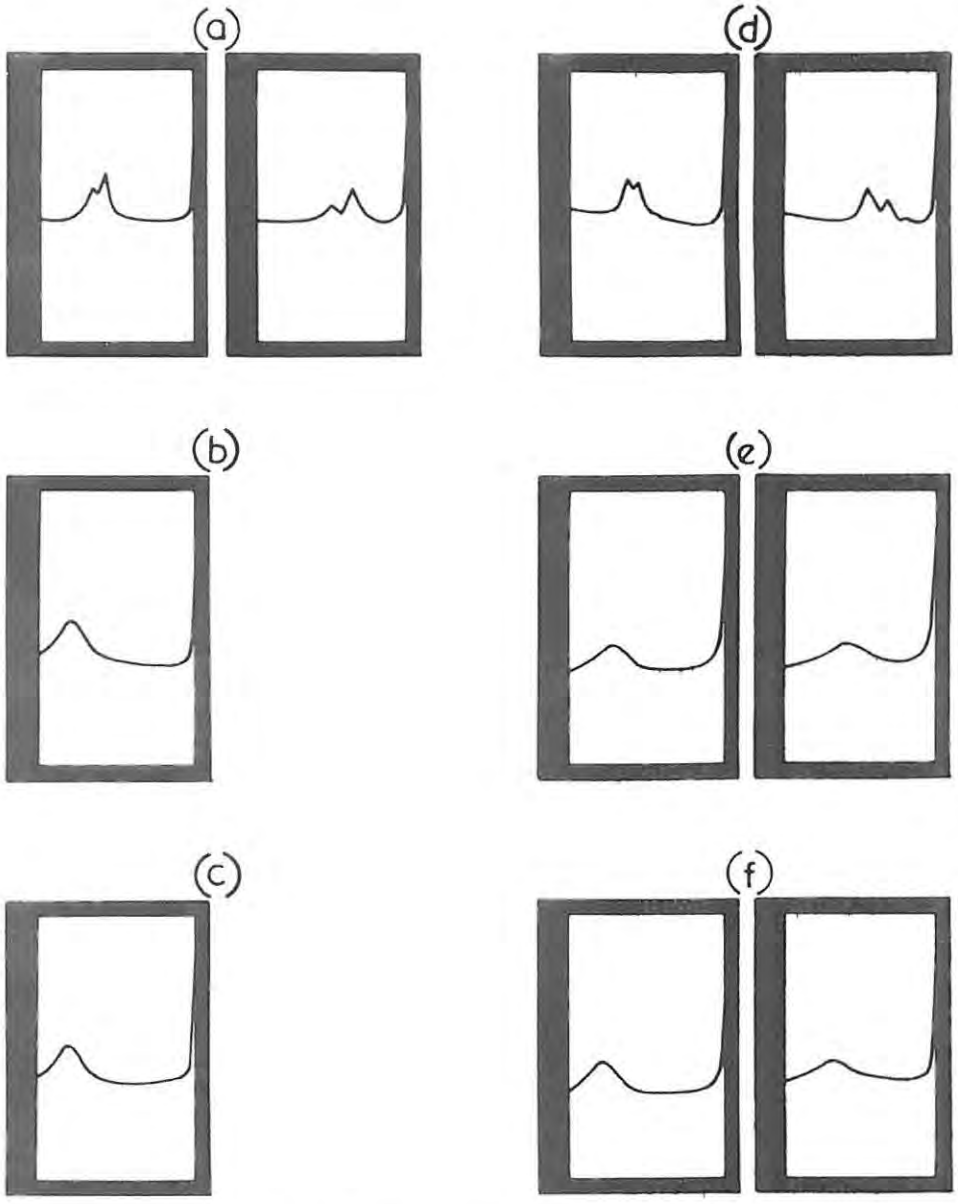

FIG. 34. Sedimentation patterns of (a) thermally denatured acid-soluble collagen, (b) thermally denatured acid-soluble collagen +5Mrads, (c) thermally denatured acid-soluble collagen + 10 Mrads, (d) thermally denatured neutral-salt-soluble collagen, (e) thermally denatured neutral-salt- soluble collagen + 5 Mrads, (f) thermally denatured neutral-salt-soluble collagen + 10 Mrads. The photographs were taken after 80 & 120 min. at 56100 rev./min. & 35°. Sedimentation is from left to right.

TABLE XIII.

RATE OF COLLAGEN-FOLD FORMATION OF ACID-SOLUBLE COLLAGEN AFTER γ - IRRADIATION AND COOLING TO 15°.

Solutions of irradiated collagen were thermally denatured at 45° for 15 min before being cooled to 15°

Irradiation Dose (Mrad)	Initial rate of mutarotation at 365 mμ and 15° $(d\alpha/dt)_o$ deg/min.		Specific rotation (deg) after 48 hr at 15° $[\alpha]^{15}_{365}$	
	neutral-salt-soluble collagen	acid-soluble collagen	neutral-salt-soluble collagen	acid-soluble collagen
0	6.5	7.0	-974	-1045
1.3	2.8	5.4	-944	- 950
2.6	2.1	4.9	-905	- 927
5.0	1.2	3.1	-832	- 827
10.0	0.9	1.4	-745	- 783

Irradiation Dose (Mrad)	Initial rate of reduced viscosity recovery x 10^{-2} $(d\eta_{red.}/dt)_o$ dl/g min.		Reduced viscosity (dl/g) after 48 hr at 15°	
	neutral-salt-soluble collagen	acid-soluble collagen	neutral-salt-soluble collagen	acid-soluble collagen
0	2.5	2.0	5.68	6.76
1.3	1.0	0.9	3.98	4.08
2.6	0.9	0.8	3.27	3.59
5.0	0.3	0.3	1.49	1.65
10.0	0.2	0.2	0.86	1.04

after 48 hr for neutral-salt-soluble and acid-soluble collagen are very similar. The effects of γ - irradiation on the mutarotation of acid-soluble collagen are illustrated in Fig. 35. These results show that γ - irradiation decreases the initial rate of mutarotation and the amount of helix formation in the partial re-formation on cooling of the collagen fold or some modification of this structure (Harrington and von Hippel, 1961 ; Veis, 1964).

A parallel study was also made of the change in reduced viscosity at 15° (Table XIII). Again, both neutral-salt-soluble and acid-soluble collagen gave very similar results. The effects of γ - irradiation on the recovery of reduced viscosity for acid-soluble collagen are illustrated in Fig. 36. This caused a decrease in the initial rate of viscosity recovery, $(\eta)_{red.}/dt)_o$, and the amount of chain association occuring parallels the regain in helical content.

Fig. 37 illustrates the relationship between reduced viscosity on cooling for 48 hr at 15° and the irradiation dose offered. This relationship is thus a measure of aggregation or inter-chain association (Harrington and von Hippel, 1961) which is directly related to irradiation damage. The main effect of irradiation on viscosity recovery would appear to be almost complete at a dose level of 5 Mrad, indicating that the first breaks in the polypeptide chain are more effective than subsequent breaks in reducing subunit chain association. The almost linear relationship between specific rotation and irradiation dose is also

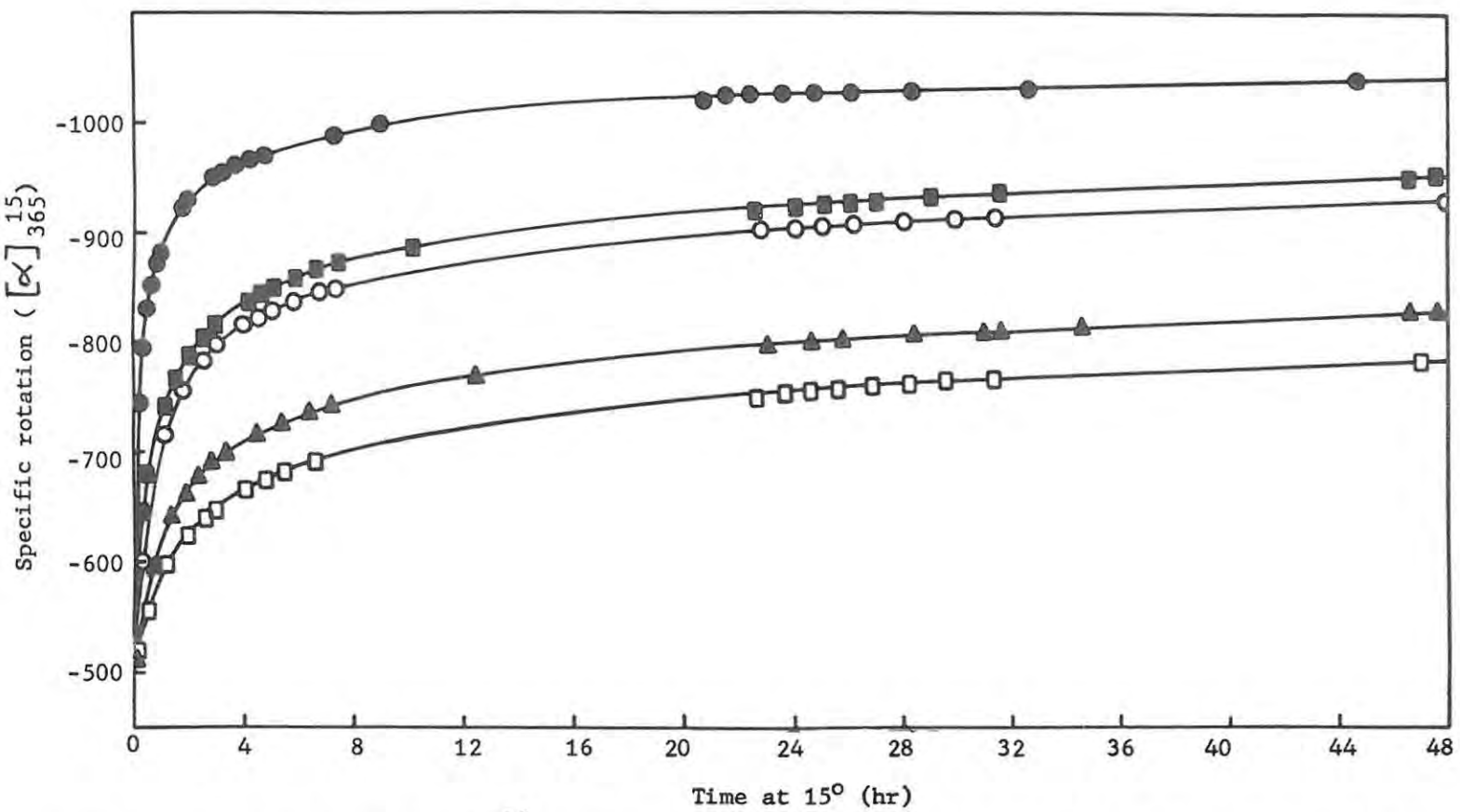

FIG. 35 Specific rotation ($[\alpha]_{365}^{15}$) as a function of time of γ - irradiated acid-soluble collagen. Solutions (1.3mg /ml) were thermally denatured at 45° and then kept at 15°. ● , not irradiated; ■ , 1.3 Mrad ; O , 2.6 Mrad ; ▲ , 5 Mrad ; □ , 10 Mrad .

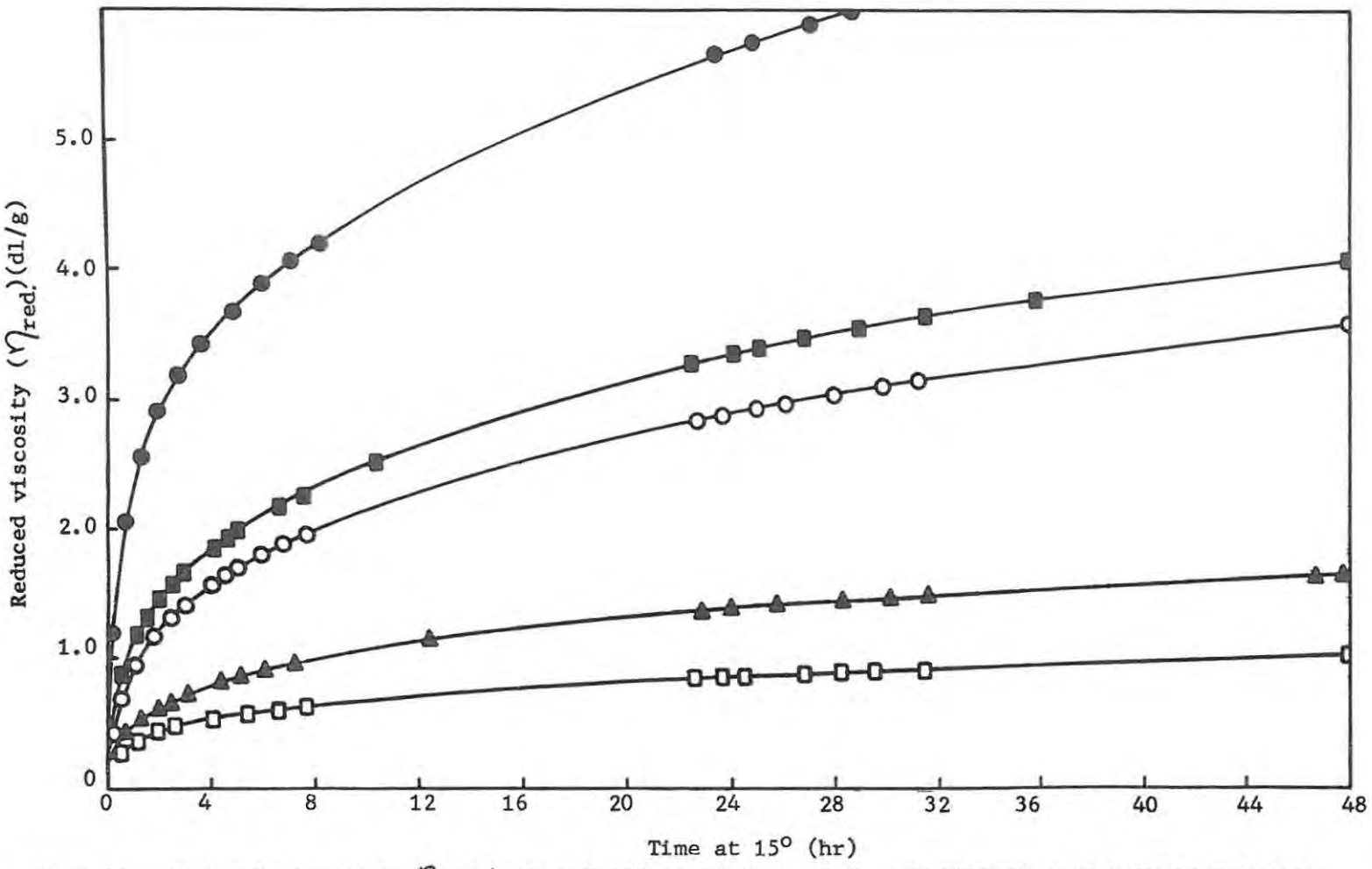

FIG. 36 Reduced viscosity ($\eta_{red.}$) as a function of time of γ - irradiated acid-soluble collagen. Solutions (1.3 mg /ml) were thermally denatured at 45° and then kept at 15°. ● , not irradiated ; ■ , 1.3 Mrad ; ○ , 2.6 Mrad ; ▲ , 5 Mrad ; □ , 10 mrad .

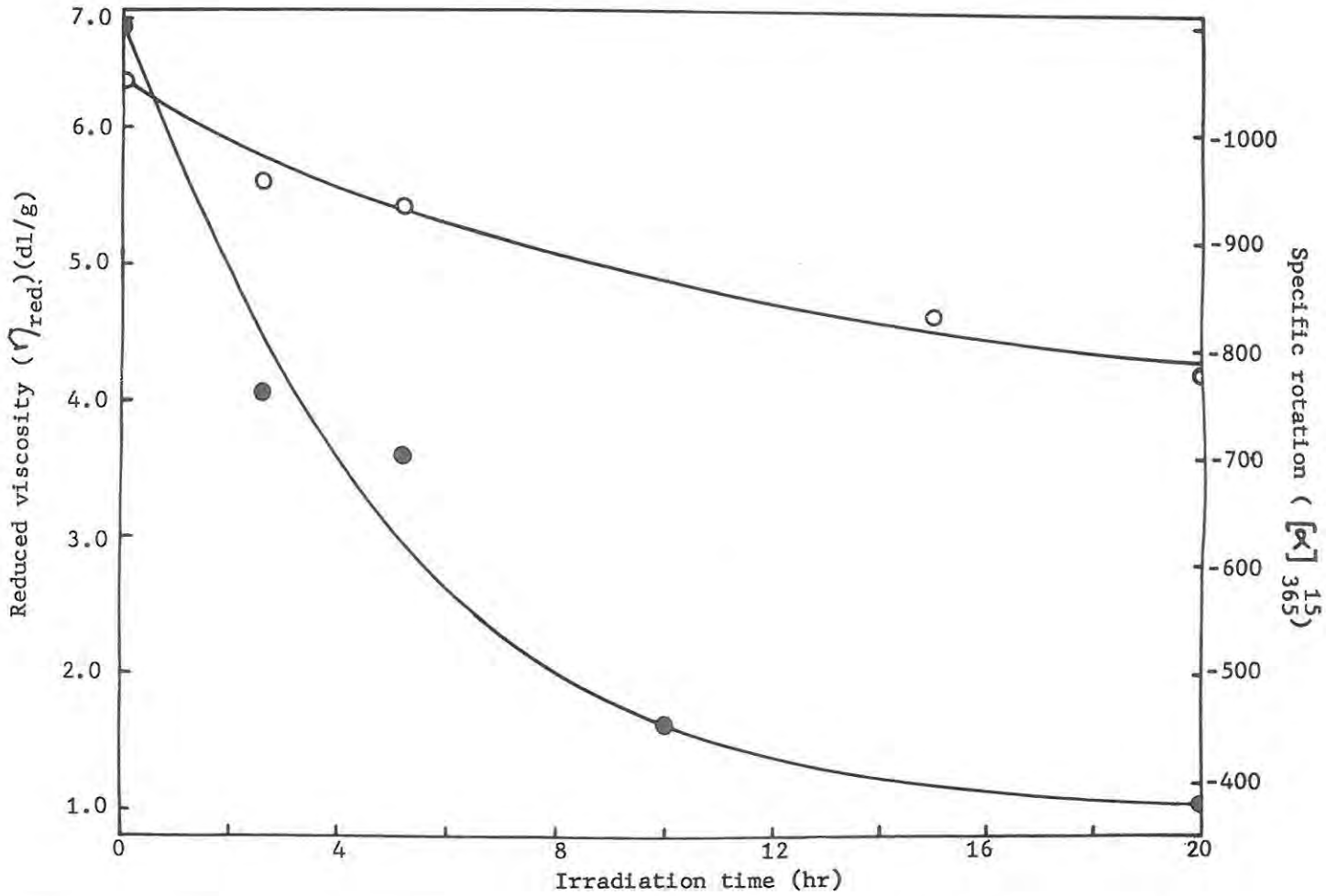

FIG. 37. Specific rotation (O) & reduced viscosity (●) as functions of γ - irradiation dose for irradiated acid- soluble collagen. Solutions (1.3 mg /ml) were thermally denatured at 45° and then kept at 15° for 48 hrs.

illustrated in Fig. 37.

In order to investigate the order of reaction for the recovery of optical rotation and reduced viscosity at 15°, with and without irradiation, log ($d[\alpha]/dt$) and log ($d\eta_{red.}/dt$) were plotted against time. Fig. 38 illustrates a typical relationship indicating that the coil-to-helix reaction is not a first-order reaction (Piez and Carrillo, 1964 ; Harrington and von Hippel, 1961).

It is apparent that the present study bears a close resemblance to earlier studies using ultraviolet irradiation discussed in Chapter IV (Cooper and Davidson, 1966 ; Davidson and Cooper 1967 a).

Amino acid analysis.

In Table XIV the amino acid analysis of irradiated neutral-salt-soluble and acid-soluble collagen are given. Comparison of these values with those reported for non-irradiated neutral-salt-soluble and acid-soluble collagen (Cooper and Davidson, 1965) show that little if any destruction of amino acids results at the prescribed levels of irradiation.

Components 1, 2, 3 and 6, 7, 8.

Components 1, 2 and 3 were separated by chromatography from the rest of the collagen, amalgamated and freeze dried. This material was then hydrolyzed for 24 hr at 100° under vacuum in sealed tubes with

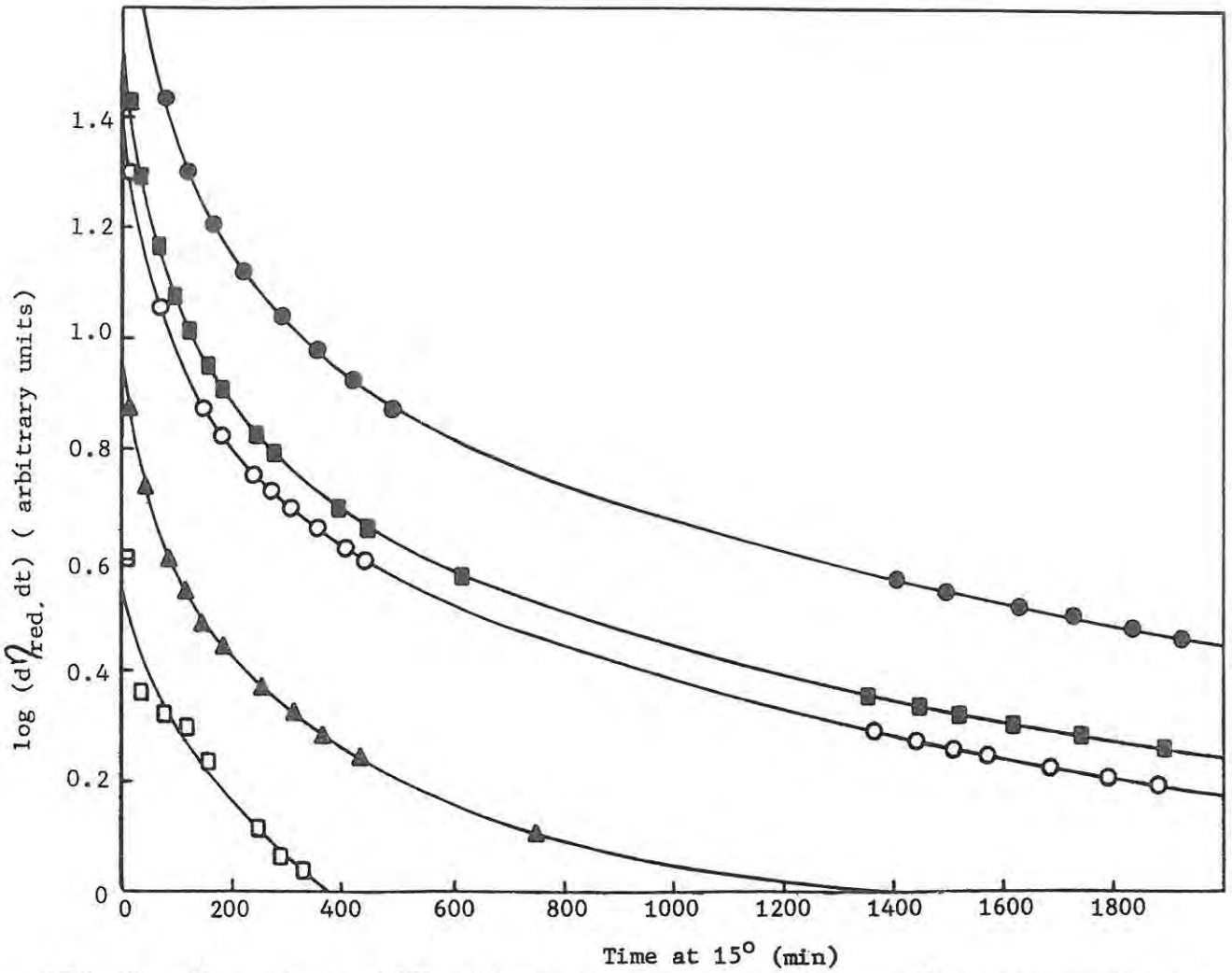

FIG. 38 Plots of log ($d\eta_{red.}$/dt) as a function of time at 15°for solutions of γ - irradiated acid-soluble collagen. Solutions (1.3 mg /ml) were thermally denatured at 45°. ● , not irradiated ; ■ , 1.3 Mrad ; ○ , 2.6 Mrad ; ▲, 5 Mrad ; □ , 10 Mrad .

TABLE XIV.

AMINO ACID COMPOSITION OF γ - IRRADIATED SOLUBLE COLLAGEN.

Amino Acid	Amino acid composition (g/100 g of protein)			
	Neutral-Salt-Soluble Collagen		Acid-Soluble Collagen	
	5 Mrad	10 Mrad	5 Mrad	10 Mrad
Ala	8.15	7.86	8.42	8.62
Arg	7.38	7.26	7.58	7.70
Asp	6.41	6.95	6.50	6.33
Glu	9.64	10.39	9.64	9.69
Gly	18.89	18.02	19.76	20.29
His	0.59	0.75	0.57	0.56
Hyl	0.84	0.78	0.89	0.58
Hyp	11.59	10.52	11.61	11.80
Ile	1.59	1.89	1.57	1.62
Leu	2.73	3.21	2.72	2.82
Lys	3.26	3.71	3.23	3.21
Met	0.54	0.71	0.42	0.78
Phe	1.76	1.95	1.78	1.81
Pro	11.21	10.52	11.57	11.89
Ser	3.37	3.52	3.39	3.48
Thr	1.86	2.04	1.77	1.81
Tyr	0.49	0.72	0.47	0.48
Val	2.08	2.34	2.09	2.13
TOTAL	93.16	94.05	94.82	96.44

6N-HCl. Components 6, 7 and 8 were similarly separated, amalgamated and dialyzed against distilled water before being freeze dried and hydrolyzed at 100°. About 0.3 mg of components 1, 2, 3 and about 3.6 mg of components 6, 7, 8 were prepared in this way. These preparations were then analyzed by two dimensional high voltage electrophoresis and chromatography. Repeat runs showed that components 1, 2 and 3 contained no amino acids detectable by the method used, but that ninhydrin-positive material remained at the origin after electrophoresis and migrated in a long streak after chromatography. Components 6, 7 and 8 were shown to contain lysine (++), arginine (++), histidine (+++), alanine (+++), glycine (++), serine (+), valine (+), leucine (+), glutamic acid (++) and aspartic acid (++), where the plus signs indicate the intensity of the spots as detected visually. In addition, ninhydrin-positive material not corresponding to amino acids and similar to that found in components 1, 2 and 3, was also present.

The quantitative determination of amino acids was also carried out on the above hydrolyzed preparations (Table XV). In the case of components 1, 2 and 3, both aspartic and glutamic acids as well as tyrosine and phenylalanine are present in relatively high amounts. The presence of these amino acids would explain both the acidic and aromatic character of these components. In the case of components 6, 7 and 8 the hydroxyproline and hydroxylysine contents

TABLE XV.

AMINO ACID COMPOSITION OF COMPONENTS 1, 2, 3 and 6, 7, 8.

Amino Acid	Amino Acid composition (residues/1000 residues).		
	Components 1,2,3.	Components 6,7,8.	Components 6,7,8 less collagen fraction.
Ala	44	105.0	89.6
Arg		48.7	51.5
Asp	122	48.4	36.7
Cys		NIL	NIL
Glu	191	97.1	135.2
Gly	263	313.0	284.8
His		11.0	22.3
Hyl		5.0	3.7
Hyp		63.5	NIL
Ile		13.2	12.1
Leu		24.5	25.2
Lys	<10	35.1	51.5
Met		0.6	NIL
Phe	72	12.3	13.1
Pro		100.8	77.0
Ser	175	79.3	152.9
Thr	<5	22.9	31.9
Tyr	130	5.9	10.7
Val		13.7	1.6

strongly suggest the presence of a collagen fraction. Since it is reasonable to assume that all the hydroxyproline present is contained in a fraction having a typical collagen amino acid analysis, the composition of components 6, 7 and 8 was also expressed allowing for the presence of such a fraction (Table XV). In this way some indication as to the amino acid content of the basic telopeptides believed to be present was obtained. Lysine, histidine and tyrosine may be noted to be present in relatively high amounts explaining the basic and aromatic nature of these components.

Owing to the small amount of components 1, 2 and 3 recovered (about $\frac{1}{4}$ mg), the analysis reported in Table XV is only an approximation of the major amino acids found present in this preparation. The small size of the minor amino acid peaks made an accurate analysis difficult. Three additional ninhydrin-positive compounds common to both component preparations were also recorded but not identified. Two of these unidentified compounds eluted in front of aspartic acid (designated P and Q), and the third (designated R) just before lysine. Components 1, 2 and 3 contained about 27% compound P, about 6% compound Q together with a trace of compound R, while components 6, 7 and 8 contained about 0.3% compound Q and about 3% compound R. Both the elution positions of compounds P and Q, as well as the relatively high serine contents found present in both components 1, 2, 3 and 6, 7, 8 suggest the presence of phosphatides in these preparations (Rapport and Norton, 1962).

The nature of components 1, 2, 3 and 6, 7, 8 is again discussed in Chapter VI.

Discussion.

The effect of γ - irradiation on the subunit composition of both neutral-salt-soluble and acid-soluble collagen has indicated the labile nature of all those components showing a high aromatic absorption at 278 mμ. From this point of view both tyrosine and phenylalanine would appear to play an important role in the present study. The resultant elution of these degraded components at or near the void volume of the chromatography column would indicate that either low molecular weight components have been liberated by the irradiation, or that changes in the cationic character of particular components has taken place. The sedimentation studies show that degradation of the original collagen occured on irradiation, resulting in material with a range of molecular weights generally lower than that of the α - subunits.

In the case of the α_2-α_2 , α_1-α_1 , β_{11}-β_{11} and β_{12}-β_{12} dimeric subunits (see also Chapter III), their labile nature may be expected as tyrosine is thought to play a significant role in the intermolecular linkage of such subunits (Rubin et al. 1963). The absence of the β - peak indicated by the ultracentrifugation of irradiated collagen would also suggest that depolymerisation of the major α_2-α_2 and α_1-α_1 dimers takes place.

The chromatography and sedimentation data discussed above show that γ - irradiation doses in the range 1.3 to 10 Mrad bring about considerable change in the subunit composition of both types of soluble collagen. Yet the mutarotation data shows that even after 10 Mrad of irradiation the collagen still undergoes considerable mutarotation to give relatively high specific rotations. Thus after heat-denaturation and storing for 48 hr at 15° unirradiated acid-soluble collagen has specific rotations of $[\alpha]_{365}^{15} = -1045^{\circ}$ and $[\alpha]_{D}^{15} = -324^{\circ}$, while acid-soluble collagen after 10 Mrad of irradiation has specific rotations of $[\alpha]_{365}^{15} = -785$ and $[\alpha]_{D}^{15} = -251^{\circ}$ under the same conditions of mutarotation. The latter values are very much higher than the mean residue rotation of collagen in the denatured form ($[\alpha]_{365}^{15} \simeq -450^{\circ}$, $[\alpha]_{D}^{15} \simeq -90^{\circ}$ to -120°), and show about 75% recovery in optical rotation when compared with un-irradiated collagen. At the lowest level of irradiation used here, the chromatography data showed a considerable change in the subunit structure but the mutarotation studies reveal that the collagen still reforms the helical structure to a large extent, as assessed from the high specific rotation ($[\alpha]_{365}^{15} = -952^{\circ}$, $[\alpha]_{D}^{15} = -315^{\circ}$) after irradiation and mutarotation at 15° for 48 hr. This corresponds to a 95% recovery in helical content.

In contrast to the above findings, the recovery of reduced viscosity after γ - irradiation, heat-denaturation and storage at 15°

for 48 hr shows relatively low recoveries. As shown in the data presented above, after 10 Mrad of irradiation the viscosity increases by only a very small amount after 48 hr at 15°. It can therefore be concluded that even after 10 Mrad of irradiation followed by thermal denaturation in solution, collagen still reforms into a largely helical structure. This mutarotation data would suggest that crystalline portions of the collagen triple helix are not degraded by γ - irradiation at the prescribed levels of irradiation. This is also supported by evidence from sedimentation and amino acid analysis. The viscosity data would indicate that these crystalline portions are no longer capable of association to give the native molecule.

On the basis of the above data it is concluded that for neutral-salt-soluble and acid-soluble collagen in the dry state, peptide bond fission occurs without significant destruction of amino acids, during γ - irradiation at dose levels between 1.3 and 10 Mrad. The amount of peptide bond fission is sufficient to lower the reduced viscosity of the irradiated collagen significantly, but does not reduce the helical content to the same degree. In view of the rapid depolymerisation of the dimeric subunits (component 5 of Fig. 29), it is suggested that the initial site of action of γ - irradiation is the telopeptide residues. These contain a high proportion of aromatic amino acids (Rubin et al. 1963) and are considered to be responsible for the formation of these dimers (Davidson and Cooper, 1967 b) and the β-

subunits (Drake et al. 1966). As the irradiation dose is increased, peptide bond fission is the next stage in the reaction. In many respects the action of γ - irradiation appears to be similar to that of ultraviolet irradiation (Cooper and Davidson, 1965, 1966 ; Davidson and Cooper, 1967 a).

Several authors (see for example, Cassel, 1959) have observed an increase in optical absorption in the region of 280 mμ after γ - irradiation of proteins, which has been attributed to the formation of ultraviolet-absorbing complexes. The relatively large absorption at 278 mμ of the main chromatographic component found after irradiation in the present study may in part be due to this.

CHAPTER VI.

THE INTERMOLECULAR RELATIONSHIP BETWEEN NEUTRAL-SALT-SOLUBLE AND ACID-SOLUBLE COLLAGEN.

Introduction.

Soluble collagen may be extracted from collagenous tissue with such solvents as neutral salt solutions and weak acids. While the resulting extracts display many similar physical and chemical characteristics, they differ significantly with respect to their solubility properties. Neutral-salt-soluble collagen has been shown to be the precursor collagen (Harkness et al. 1954 ; Mazurov and Orekhovitch, 1960), while acid-soluble collagen is the older fibre-forming protein (Jackson and Bentley, 1960). It has also been suggested (Jackson, 1953) that there is no sharp division between acid-soluble and insoluble collagen as indicated by the increased solubility in acid, of tendon treated with hyaluronidase.

A topic which has long been the subject of some dispute, is the relative proportions of the α- and β - subunits in denatured soluble collagens. It would seem important to establish this relationship before attempting to relate neutral-salt-soluble and acid-soluble collagen. A further important point that has been neglected in some cases in the past, is the removal of neutral-salt-soluble collagen prior to the extraction of acid-soluble collagen.

If neutral-salt-soluble collagen is not initially removed, then the proportions will relate to a mixture in the case of acid-soluble collagen.

Although it has been demonstrated that neutral-salt-soluble collagen may in some instances yield only α- subunits on thermal denaturation (Jackson, 1962 ; Piez et al. 1961), it has also been shown that similar preparations may yield considerable quantities of β- subunits (Mazurov and Orekhovitch, 1960 ; Piez et al. 1960 and 1961 ; Orekhovitch et al. 1960 ; Jackson, 1962 ; Heidrich and Winston, 1965). Nänto et al.(1963) using starch gel-electrophoresis in fact found no differences in the subunit compositions of neutral-salt-soluble and acid-soluble collagens prepared from the same source. Likewise, the present investigations using ultraviolet irradiation (Cooper and Davidson, 1965, 1966 ; Davidson and Cooper, 1967a) and γ- irradiation (Davidson and Cooper, in press) have indicated no significant differences between neutral-salt-soluble and acid-soluble collagen as currently prepared (see Chapters IV and V). It is generally considered, however, that the thermal denaturation of acid-soluble collagen yields larger proportions of β - subunits than does neutral-salt-soluble collagen. It would appear then, that largely on this basis, the current intramolecular relationship between acid-soluble and neutral-salt-soluble collagen has been formulated. Although such a mechanism has been shown to take place in the biosynthesis of insoluble collagen, it does not satisfactorily explain the large solubility differences

displayed by neutral-salt-soluble and acid-soluble collagen (see summary by Harding, 1964).

If the above supposition is correct, then the evidence relating to the intra or intermolecular relationship of acid-soluble and neutral-salt-soluble collagen must be reviewed. A more detailed study as to the nature of neutral-salt-soluble collagen, as well as the methods of preparation would appear to explain many of the contradictory findings regarding the relationship between neutral-salt-soluble and acid-soluble collagen.

Neutral-Salt-Soluble Collagen.

A closer investigation into the methods currently used to extract and prepare neutral-salt-soluble collagen (Piez et al. 1963), will reveal that significant changes in the physicochemical properties of the collagen extract take place during preparation. The physicochemical properties of the initial neutral-salt extract prepared under neutral pH conditions (Fessler, 1960 a and b) would appear to be very different from the properties displayed after such extracts have been purified using dilute acetic acid. In order to illustrate these differences, neutral-salt-soluble collagen was prepared under strictly neutral conditions (preparation No. 3), and compared with the neutral-salt-soluble collagen preparations as currently prepared and purified using dilute acetic acid (preparations No. 1 and 2). The extraction

details for the above collagen preparations are presented in Chapter II.

The following observations were made:

1. The preparations purified using dilute acetic acid (No. 1 and 2) were completely insoluble in 5% NaCl, while the preparation prepared under neutral conditions (No. 3) was almost completely soluble in 5% NaCl.
2. Preparations No. 1 and 2 were completely soluble in 0.15 M-HAc, while preparation No. 3 was almost completely insoluble in 0.15 M-HAc.
3. Preparations No. 1 and 2 displayed melting points in 0.06 acetate buffer (pH 4.8) in the region of 38°. These melting points were measured viscometrically (see Chapter III). Preparation No. 3 on the other hand remained insoluble in the same 0.06 acetate buffer even when actually boiled for 2 hours. The shrinkage temperature of this insoluble precipitate was also measured by the capillary tube method of Brown et al. (1958). While no melting of the precipitate was recorded below 100°, further evidence would be required to verify such a high shrinkage temperature.
4. Preparation No. 3 in IN-NaCl was observed to gel on heating at 37°. Gelling was also observed to take place at the capillary orifice when taking viscosity measurements at 20°. Preparations

No. 1 and 2 displayed none of these characteristics as they were not soluble in IN-NaCl.

5. While preparations No. 1 and 2 were very white in colour, preparation No. 3 was inclined to be brown. This brown colouring increased with the time taken in extracting the original skin with 10% NaCl indicating the occlusion of impurities.

6. Table XVI illustrates the amino acid compositions of preparations No. 1 and 3. Preparation No. 1 prepared under acid conditions has a typical collagen analysis (Piez et al. 1963). Preparation No. 3 on the other hand displays several anomalies when related to a typical collagen analysis: hydroxyproline and glycine are low indicating the presence of a large non-collagenous fraction ; lysine ; histidine and tyrosine are excessively high while phenylalanine, methionine, threonine, isoleucine and leucine are high. The excessive amounts of lysine, histidine, tyrosine and phenylalanine present in preparation No. 3 would certainly suggest the presence of the telopeptide components 1, 2, 3 and 6, 7, 8 discussed in Chapter V. Furthermore, the unidentified compounds P and Q reported to be present in components 1, 2, 3 and 6, 7, 8 were also found to be present in preparation No. 3. The low recovery of total amino acids recorded for preparation No. 3 (67%) would also suggest the presence of a large carbohydrate fraction which may or may not be present as an occluded impurity.

TABLE XVI.

AMINO ACID COMPOSITION OF NEUTRAL-SALT-SOLUBLE CALF-SKIN COLLAGEN.

Amino Acid	Amino Acid Composition (residues/1000 residues)	
	Collagen prepared by acid purification	Collagen prepared under neutral conditions.
Ala	109.0	96.4
Arg	44.9	61.6
Asp	52.8	71.5
Cys	NIL	NIL
Glu	71.0	96.2
Gly	314.6	178.7
His	4.1	13.8
Hyl	5.5	2.7
Hyp	96.6	34.1
Ile	13.3	28.1
Leu	22.9	61.7
Lys	24.2	83.1
Met	3.9	11.6
Phe	11.3	23.3
Pro	109.7	76.2
Ser	34.0	55.0
Thr	16.8	47.1
Tyr	2.9	18.7
Val	20.0	40.2
% Recovery	93.2	66.6

7. The serum-like proteins extracted from calf-skin with 10% NaCl were all found to be extremely soluble in water (Cooper, Russell and Davidson, 1967). This would suggest that the non-collagenous fraction associated with the collagen present in preparation No. 3, is present either as an occluded impurity or as part of the native tropocollagen monomer (see below). Such material may form part of the ground substance reported to be associated with collagen (Steven and Jackson, 1967).

These latter observations serve to illustrate the very different physicochemical characteristics displayed by neutral-salt-soluble collagen which are related directly to the methods of preparation. As neutral-salt-soluble collagen has been shown to be the precursor collagen, the possibility that this soluble collagen extract is in fact monomeric tropocollagen must be considered. In the present text the term monomeric tropocollagen refers to the native monomer with intact terminally located telopeptide side-chains. It is a reasonable supposition (Schmitt et al. 1964 ; Hodge and Schmitt, 1958 ; Schmitt 1956) that side chains rich in acidic residues are present at the one end of the molecule inducing a small negative charge, while side-chains rich in basic residues are present at the other extremity of the molecule inducing a small nett positive charge. A number of workers (Rubin et al. 1963 ; Bornstein et al. 1966 ; Drake et al. 1966) have isolated telopeptides rich in both acidic and aromatic amino acids,

while the isolation of components 1, 2, 3 and 6, 7, 8 carried out in the present study, would suggest the presence of both acidic and basic telopeptide side-chains (Cooper and Davidson, in press). Because of the failure of workers to report on the presence of basic telopeptides in soluble collagens prepared using exhaustive acetic acid extraction procedures, it would suggest that such telopeptides are labile under acid conditions. The ease with which mature fish-skin collagen may be taken up in very dilute acetic acid would suggest a depolymerisation mechanism resulting from the presence of acid-labile telopeptides. Conversely, the presence of acidic telopeptides labile towards alkaline conditions would explain the depolymerisation induced by such conditions (Courts, 1960).

Evidence for such an intermolecular relationship between neutral-salt-soluble and acid-soluble collagen involving labile telopeptide linkages is presented below.

Discussion.

Although the currently accepted intramolecular mechanism relating neutral-salt-soluble and acid-soluble collagen has served as a foundation upon which further investigation may be based, it is felt that such a relationship alone is insufficient to explain the very different solubility properties displayed by these two forms of collagen. It is therefore proposed that neutral-salt-soluble and

acid-soluble collagen are related on an intermolecular basis in their native environment, but that their subsequent extraction and preparation as currently carried out under mildly acid and basic conditions, results in the degradation of terminally located telopeptides, the presence of which play a vital role in the physicochemical properties displayed by such soluble collagen preparations.

When compared with acid-soluble and neutral-salt-soluble collagen preparations as currently prepared using dilute acetic acid, the very different characteristics displayed by neutral-salt-soluble collagen prepared under neutral conditions, would appear to have been overlooked by many workers in this field. Fessler (1960 a and b) would appear to be one of the few workers to have reported on the physicochemical properties of such neutral-salt extracts.

The initial ability of such neutral-salt-soluble collagen to form insoluble polymeric material as well as to gel at body heat would strongly suggest an intermolecular polymerisation mechanism involving telopeptide linkages. Furthermore, the extraction of the initial collagen precipitate with dilute acetic acid (the method currently used in preparing neutral-salt-soluble collagen) results in a soluble collagen fraction incapable of undergoing vigorous polymerisation or gelation at body heat. The progressive depolymerisation of the initial collagen precipitate under weakly acid conditions resulting in the loss of telopeptide side-chains would explain such findings.

When the initial solubility of neutral-salt-soluble collagen is compared with that of the peptideless monomer prepared by Davison and Drake (1966) it may be seen that the latter displays solubility over the whole pH range, except between pH 9-11. A major shift in the isoelectric point from about 5 (acid-soluble collagen) to about 10 would appear to have taken place. But for the presence of telopeptide side-chains, native neutral-salt-soluble collagen would appear to be similar to the above monomeric collagen. Because of the presence of these telopeptide side-chains, however, intermolecular polymerisation of the native monomer takes place on lowering the pH resulting in an insoluble polymeric material. The inability of acid-soluble collagen to form such a polymeric precipitate under these conditions would be difficult to explain on an intramolecular basis.

If native neutral-salt-soluble collagen is the tropocollagen monomer, then the dimeric character of acid-soluble collagen must be considered. Acid-soluble collagen prepared by repeated phosphate precipitation under alkaline conditions, has been fractionated on CM-cellulose yielding the α_3, α_1, β_{11}, β_{12} and α_2 subunits (Piez et al. 1963 ; Piez, 1964). However, it must be noted that acid-soluble collagen prepared using no phosphate precipitation has been shown to yield major unknown components (Schleyer, 1962 ; Hollmèn and Kulonen, 1964 ; Francois and Glimcher, 1965 ; Davidson and Cooper, 1967 b ; Tristram et al. 1965). Davidson and Cooper (1967b) have related four

such components to the $\alpha_1\alpha_1$, $\alpha_2\alpha_2$, $\beta_{11}\beta_{11}$ and $\beta_{12}\beta_{12}$ dimeric subunits respectively, and attribute their presence to the dimeric nature of the parent material. Such a dimeric parent material would explain the β_{22}- subunit reported by Bornstein et al. (1964), as well as the presence of only β - subunits in an acid-soluble extract reported by Bakerman and Hersh (1964). In view of these findings the latter workers concluded that "the molecular weight of the undenatured soluble collagen monomer must be a whole-number multiple of the molecular weight of the β - chain". This conclusion would only be justified if the parent material was in fact dimeric.

The presence of dimeric subunits in solutions of denatured collagens immediately stresses the need for extreme caution in the interpretation of sedimentation data. Davidson and Cooper (1967b) have shown that the major $\alpha_1\alpha_1$ and $\alpha_2\alpha_2$ components sediment with the β - subunits, and suggest that the minor $\beta_{11}\beta_{11}$ and $\beta_{12}\beta_{12}$ components (molecular weights approximately 350,000) may be incorrectly characterized as γ - components. A molecular weight of 260,000 for the collagen monomer (Davison and Drake, 1966) as well as the wide range of molecular weights and α/β ratios reported in the literature would support such findings. Furthermore, such evidence immediately casts doubt as to the labile nature of the β - subunit under certain prescribed conditions when using evidence from sedimentation (Davidson and Cooper, 1967a ; Drake et al. 1966 ; Chun and Doty, 1958 ; Astrup et al.

1958 ; Doty and Nishihara, 1958). Mazurov and Orekhovitch (1960), in fact find no degradation of the β - subunit at pH 9 after first isolating the subunit using a chromatography procedure. The depolymerisation of the $\alpha_1\alpha_1$ and $\alpha_2\alpha_2$ components may in some instances explain such findings.

Evidence from intrinsic viscosity measurements is also worthy of mention. While numerous values for the intrinsic viscosity of collagen appear in the literature (see summary by Kahn and Witnauer, 1966), the absence of values extrapolated to zero rates of shear make any comparison difficult. Davison and Drake (1966) quote a value of 10 dl/g for pronase treated monomeric tropocollagen at zero rate of shear, and attribute their higher values for untreated acid-soluble collagen (17-20 dl/g) as being due to the presence of linear polymers. Kahn and Witnauer (1966) quote a value of 27 dl/g for acid-soluble collagen at low rates of shear, which would support the theoretical supposition that the collagen dimer is terminally linked with a 10% overlap (Hodge and Petruska, 1963), and would display an intrinsic viscosity of approximately 30 dl/g. This evidence would also suggest that acid-soluble collagen is dimeric.

When related to the current methods of extracting soluble collagens, much may be explained using the above hypothesis. Firstly, the preparation of neutral-salt-soluble collagen would initially yield an active monomer which when acidified would yield an insoluble polymer

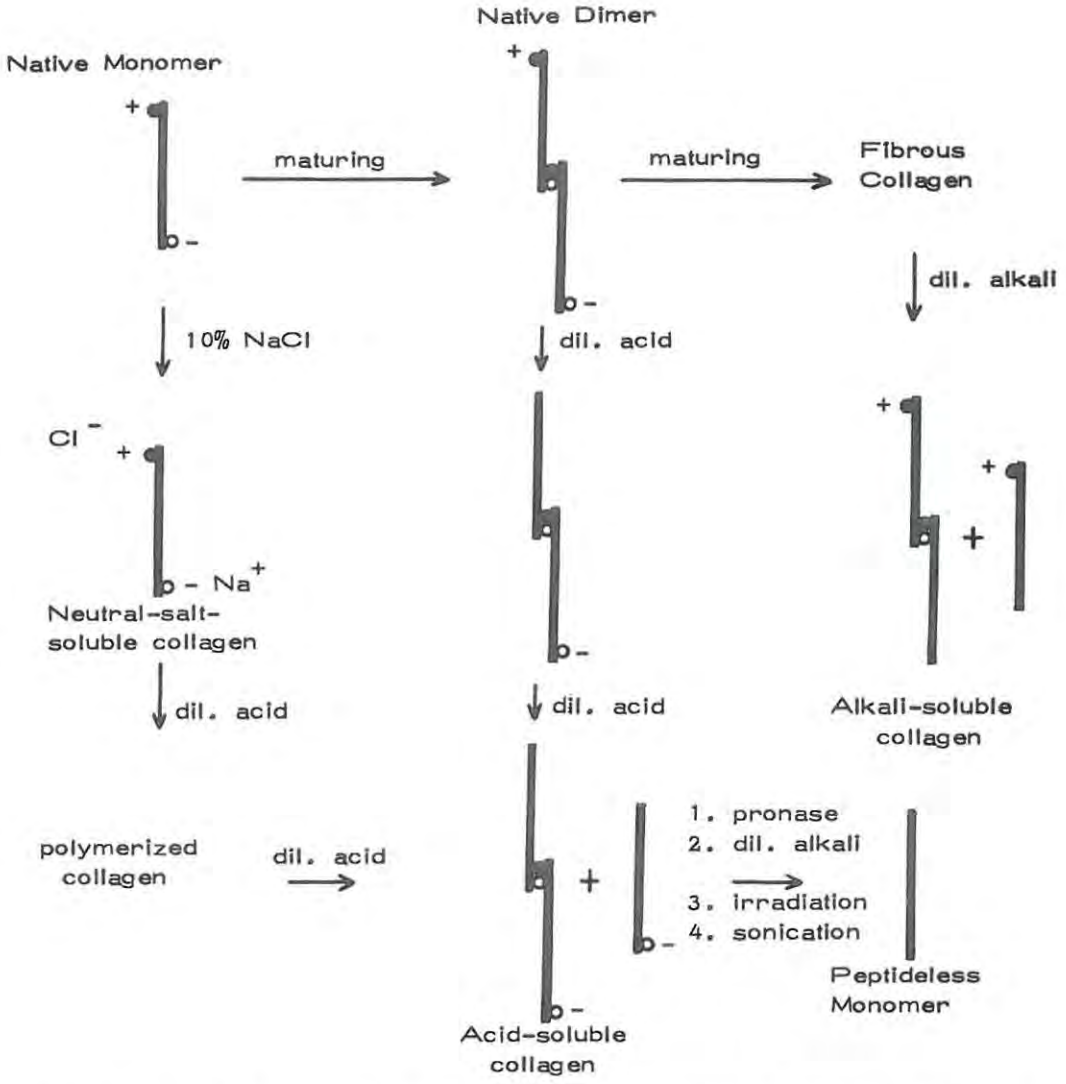

FIG. 39. The Intermolecular Relationship Between Soluble Collagens.

(Fig. 39). Subsequent extraction with dilute acetic acid would yield a mixture of soluble inactive monomer and inactive dimer, which on further purification would result in the progressive enrichment of inactive monomeric collagen. Secondly, the preparation of acid-soluble collagen after the prior removal of neutral-salt-soluble collagen would result in an initial extract of inactive dimeric collagen which on further purification would be progressively enriched with inactive monomeric collagen. It may be readily seen that in both cases the resultant monomer-dimer content will depend on the number of purification stages carried out, as well as the pH conditions used. Furthermore, these conclusions would indicate that native monomeric tropocollagen may only be prepared in a neutral medium of high ionic strength. Such a medium would effectively smother any electrostatic interaction of the highly active collagen monomer, and so inhibit polymerisation (Fig. 39).

The term linear polymerisation would appear to explain the well ordered mechanism of fibre formation from native tropocollagen monomers, while the term aggregation may be applied to those preparations in which the partial or complete destruction of telopeptide side-chains has taken place, resulting in an impaired ability of such preparations to form native fibres.

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