NATIONAL BUREAU OF STANDARDS REPORT

NBS PROJECT		NBS REPORT
311.05-11-3110561	June 30, 1970	10 271

Progress Report

on

AGGREGATION PHENOMENA OF COLLAGEN

by

James M. Cassel

Supervisory Chemist, Dental Research Section, National Bureau of Standards, Washington, D. C. 20234.

This investigation is part of the dental research program conducted by the National Bureau of Standards in cooperation with the Council on Dental Research of the American Dental Association; the National Institute for Dental Research; the Dental Research Division of the U. S. Army Medical Research and Development Command; the Dental Sciences Division of the School of Aerospace Medicine, USAF; and the Veterans Administration.

U.S. DEPARTMENT OF COMMERCE

NATIONAL BUREAU OF STANDARDS

Aggregation Phenomena of Collagen

I. Introduction

II. The Collagen Solution

A. Aspects of Solubilization Procedures

B. Non-collagen-like Components

III. Fibrillar Forms Derivable from Dilute Collagen Solutions

IV. Kinetics of Collagen Precipitation

A. Fibril Formation as a Nucleation and Growth Process

B. Role of Polyanions in Collagen Precipitation

V. Some Thermodynamic Aspects of Collagen Aggregation

A. Phase Diagram Determinations

B. Hydrophobic Bonding Aspects

C. Time-Dependent Changes in the Reversibility of Native-Type Fibril Formation

VI. Collagen Aggregation *in vivo*

I. INTRODUCTION

Collagen, in the form of insoluble fibers or fibrous aggregates, represents the major proteinaceous constituent of most vertebrates and many invertebrates. The properties of collagen fiber are closely related to the nature and mode of attachment of its constituting subunits. That the collagen fiber represents an end product in a series of interrelated steps is obvious. That this process begins extracellularly with a macromolecular unit of rather well characterized dimensions has been recognized only since the early nineteen fifties.

An early indication that insoluble collagenous tissue was indeed derived in some fashion from a more soluble precursor came from the observation by Zachariades [1] that tendon of rat tail, suspended in very dilute formic or acetic acid, swelled markedly and then began to dissolve. This dissolution of collagenous tissue, particularly the tail tendon of rat, was studied in great detail by other French workers, Nageotte [2-10], Leplat [11-12] and Faure-Fremiet [13-14].

On dialyzing the collagen solution against distilled water, a gel was formed if the pH was above 5.0 [5,15]. Addition of sodium chloride in high concentration produced a massive precipitation, but at low ionic strengths the precipitation was in the form of fibrils, the size and shape of which appeared to vary with salt concentration and probably with pH [2-5]. Some of the fibrils had a crystalline appearance and were birefringent [2,3,13,14]. The reconstituted fibrils closely resembled the native collagen fibers observed in sections of connective tissue and their staining characteristics were those of collagenous tissue- indeed similar to those of native, intact, mammalian collagenous tissue [16]. A further development came when electron microscopy revealed the same ordered morphology, namely the repeating axial period of about 640 Å and detailed fine structure, in both the reconstituted fibrils and the native fibrils [17,18].

Early in the study of the structure of the collagen fibril, the term "protofibril" was coined [19] to connote the smallest columnar array capable of carrying the characteristics of collagen, the lateral aggregates of which were postulated to produce the collagen fibril visible in the electron microscope. Orekhovitch [20] applied the term "procollagen" to the solubilized

collagen product obtained by citrate-buffer treatment of rat skin in the belief that it was a precursor of fibrous collagen. It has since become apparent that such a definition must be restricted to certain preparations of soluble collagen. The term "tropocollagen" was applied by Gross et al.[21] to define a unit particle capable of being converted into collagen. Concurrent research[22,23] identified the tropocollagen unit to be the molecular unit of the collagen structure and not an aggregate of units which participates (in observed reactions) as a kinetic unit.

Piez[24] has observed that application of the term "tropocollagen" to soluble collagen preparations regardless of their source and nature is not completely satisfactory since the intent of the original designation was to distinguish the monomer from the fibrous form. Since "collagen" is now a better defined term meaning the protein and not the fiber, it appears preferable to use the terms "soluble collagen" or "fibrous collagen" to indicate the physical state of the collagen molecule.

The size and shape of the collagen molecule in solution has been determined by various means. Reviews of that work have been provided by Harrington and von Hipple[25], and by Veis[26]. Some representative recent measurements on native vertebrate and invertebrate collagens are provided in the review of von Hipple[27].

The common structural unit of collagen consists of three polypeptide chains (αchains) of approximately one-thousand amino acid residues each. The individual chains are in the form of left-handed helices with a pitch of about 9 Å . The triad of these is further twisted around a common axis to form a super helix with a pitch of about 100 Å (Fig. 1)[28]. The basic, rather steep, threefold, left-handed helix characteristic of these individual chains also occurs in the water-soluble form of poly-L-proline (the poly-L-proline II structure) and in polyglycine cast from an aqueous solution (polyglycine II)[29,30].

The supercoiling requires that every third residue in each chain be glycine and that the glycine residues on adjacent chains be out of phase with each other, since these positions fall along the axis of the molecule where there is no room for a side-chain (Fig. 2)[28]. The main stabilization interaction in the superhelix or tertiary stucture is derived from the cooperative system of lateral hydrogen bonds between the component polypeptide helices, whereas the secondary structure of the individual chains is main-

tained by the rigidity conferred by the rotational restrictions which exist at the α carbon to carbonyl-carbon bonds of pyrrolidine residues and the partial double-bond character of all the peptide bonds. The decreases in the denaturation temperature of acid-soluble collagen caused by addition of alcohols has been interpreted by Schnell[31] as being evidence that hydrophobic interactions also contribute to collagen intramolecular stabilization. Stabilization by means of intramolecular hydrophobic interaction does not appear likely, however, in view of the fact that projection of the amino acid side chains outward from the central axis of the extended, rigid, triple chain conformation makes it difficult to achieve close proximity of apolar residues on any adjacent pair of component α chains.

In almost all collagens analyzed[24], two of the chains appear to have essentially identical amino acid contents, a result which has led to the shorthand notation $(\alpha_1)_2\alpha_2$. The designations used to specify chains involved in intramolecular covalent crosslinking i.e., between chains of a collagen molecule are β_{11} and β_{12} for the dimeric forms and γ_{112} for the trimetric form. These dimers and trimers are chromatographically separable following denaturation and dissociation of the native helical structure.

The sequence of the amino acid residues along the rod-like unit is such that regions containing polar and apolar side chains are distributed non-uniformly giving an asymmetric or polarized structure. This distribution is assumed to play a dominant role in the modes of aggregation evidenced by the rod-like collagen units.

II. THE COLLAGEN SOLUTION

The mode of preparation and the standards of purification of such

solutions have varied with the goal of the investigation. Investigation of the amino acid composition and sequence requires removal of all detectable non-collagenous impurities. Customarily, treatment of the soluble collagen extract with relatively mild reagents is pursued until the composition of the product is altered little by additional procedure or until it approaches a predetermined standard. Final estimates of purity have been based on the extent to which known contaminants (such as hexosamine-containing polysaccharides) are minimized and on analytical values for hydroxyproline, glycine and tyrosine in particular. Studies *in vitro* formation of collagen aggregates as model systems to reveal information on ***in vivo*** synthesis require stable solutions of native collagen monomers. Comparative studies of aggregation phenomena may (but not always) require less emphasis on complete removal of impurities, but do demand a greater concern for minimizing subtle partial denaturation and for the state of dispersity in the initial system.

A. Aspects of Solubilization Procedures

The two main procedures applied to extract undenatured collagen from tissues employ dilute organic (usually citric or acetic) acids, free or in buffer form, or neutral salt solutions, and these extractions are performed in the cold. These preparations are referred to as acid-soluble and neutral salt-soluble collagen, respectively. The efficacy of the extraction procedures may be judged by (1) the similarity in amino acid composition of the soluble portion to that of the parent materials (except for relatively minor differences in tyrosine content, where differences that do exist in amino acid composition between insoluble and soluble collagens may well be a question of purification); (2) the viscosity and optical rotation of the soluble collagen solution ($[\eta] \tilde{=}$

12 - 16 dl/g; $[\alpha]_D$ = -330 to -370°); (3) the resistance of the extracted material to attack by proteolytic enzymes under conditions which in themselves do not induce loss of helical structure, and (4) the capability of the solubilized material to be reconstituted to an aggregated form which displays organization under the electron microscope very similar to that of the original parent substance.

There is considerable evidence that the ease of extraction of soluble collagen using neutral salt or weak acid solutions is determined primarily by the state of the collagen in the tissue rather than by inherent differences in the protein extracted. Gross et al.[32] proposed that the collagen extractable from connective tissue by neutral or slightly alkaline solutions of phosphates and other salts is present as a component of the ground substance, an omnipresent viscous gel of the extracellular phase of tissue. Gross[33] subsequently suggested loose, less mature fibrillar aggregates of the tissue as the source of neutral salt-extractable collagen. Such structures were presumed "young" in the sense that they had not had time to establish a more stable, sterically organized aggregate characterized by an increased number of secondary cross-links. Cold, neutral-salt solutions, being weaker dispersing agents than acidic media, are presumed to extract collagen from the more recently formed and poorly integrated fibrils whereas acid solutions solubilize collagen from older fibrils as well. By chromatographically determining the distribution of α, β and γ components in the collagen preparations from pig, rat and human skin, Piez and coworkers[34-36] have shown that the degree of intra-molecular crosslinking of acid-extracted material is significantly greater than that which has been extracted with neutral salt. Similar results with calf-skin were reported by Engel and Beier[37].

A property of soluble collagen preparations that may be expected to be of particular importance in studies of the kinetics of fibril formation is the degree of monodispersity. That collagen solutions are difficult to prepare in monodisperse form was shown by Boedtker and Doty[22], who found it necessary to apply prolonged and repeated ultracentrifugation with sifficiently dilute solutions. Solutions not treated in this way contained small amounts of aggregated material which they concluded could consist of clusters of collagen molecules or end-to-end nonlinear aggregates of collagen molecules. Additional evidence for the presence of aggregated forms in collagen preparations was provided by Hodge and Schmitt[38] who noted that ultracentrifugation significantly narrowed the distribution of the length of the collagen molecule as assessed by electron microscope observations of the segment-long-spacing (SLS) forms that are precipitated on addition of adenosinetriphosphate to acidified solutions of collagen.

Despite the conclusion of Engel and Beier[37], based on sedimentation, viscosity and light scattering measurements that there was no difference in the size and shape parameters of neutral salt-and-acid-extracted collagens, the degree of dispersity of a collagen solution appears dependent on the mode of extraction[24, 39]. The intrinsic viscosity of acid-soluble collagen is normally 10-15 percent greater than that of neutral salt-soluble material. Proteolytic enzyme attack, under non-denaturing conditions, reduces the intrinsic viscosity of the two type preparations to essentially the same value[25]. These treatments are assumed to cleave a portion of the collagen molecule at the N-terminal region which is in a non-helical configuration (approximately fifteen amino acids in each of the α chains) as well as crosslinks between adjacent collagen molecules. From their sedimentation and viscosity data, Veis and Drake[40] concluded that the

solutions prepared by weak acid extraction of fish swim bladder tunics, i.e., ichthyocol collagen preparations, contained an average of approximately 20 percent of the dimer.

Even within the collagen extracted by neutral-salt solutions, Fessler[41,42] observed a heterogeneity not explicable on the vasis of the physical methods he employed to characterize his fractions. He distinguished three fractions Fraction A, which formed fibers at 37°C that completely dissolved on cooling; fraction B, which did not precipitate at 37°C; and fraction C, which irreversibly formed fibers at 37°C. Since fraction B could be precipitated by going to higher ionic strengths, Fessler pointed out that this might indicate the presence in this fraction of an impurity whose capacity for interfering in the precipitation was reduced by the high ionic strength. Piez[24] has suggested as alternative explanations, heterogeneity in cross-linking and in the amount of lysine-derived aldehyde component, as well as the possibility of a partially denatured species, perhaps involving subtle alterations.

Candlish[43], in chromatographic experiments in which he eluted acid-soluble calfskin collagen from Sephadex columns in the presence of 3M KI (a concentration sufficient to promote complete breakdown of the triple helix), found approximately two tenths of the nitrogen applied to the column was not recovered. He assumed the Sephadex gel permitted elution of α, β and γ components only (the elution pattern suggested the presence of three components), and attributed the non-elution of a sizeable proportion to be a consequence of a spectrum of hydrogen bonded aggregates in the native soluble collagen preparation. Jackson and Bentley[44] had earlier expressed similar support for the concept that soluble collagen systems contain "a continuous spectrum of aggregates with various degrees of molecular cohesion

(or crosslinking), the degree of which depends upon the biological age of the constituted molecules." A greater degree of aggregate formation would then be expected in the extraction of the more mature collagen molecules with dilute organic acids than in the neutral-salt extraction of biologically younger material.

B. Non-Collagen-Like Components

A structural feature that has attracted considerable interest in recent years is the presence of non-collagen-like peptides, e.g., peptides having less than characteristic proportions of proline and glycine and little or no hydroxyproline, but rich in tyrosine and acidic in nature. Such peptides were revealed by proteolytic enzyme treatment of collagen preparations under conditions deemed normally not to cause loss of helical structure. Schmitt and coworkers[45-49] suggested that these peptides, termed "telopeptides", are derived from appendages that play a particular role in a number of processes including fibril formation, immunological reactions of collagen, and covalent crosslinking. The original concept[50] of native fibril formation involving the interacting, tail-like properties accounted for the formation of a columnar array of collagen units, referred to as protofibrils, as precursors to a lateral aggregation process in which neighboring protofibrils were displaced relative to one another by 1/4 of the macromolecular length (the quarter-stagger hypothesis). This concept required modification in light of electron microscope evidence that the ends of collagen molecules in the native fibril are generally separated longitudinally by several hundred angstroms[51,52].

A number of researchers[53-56] have held the view that these non-collagen-like peptides were in the category of impurities. The fibril forming abilities of collagen preparations subjected to extensive precipitation

and re-solution purification procedures were shown to be like those of preparations subjected to enzyme treatment such as trypsin, pepsin or ficin, because, provided that all traces of residual enzyme were removed, the kinetics of fibril formation as well as the morphology of the precipitated forms obtained by the two types of preparation were comparable.

On the basis of extensive sequence studies[24] Piez has suggested that, rather than multiple peptides which branch away from the helical body of the molecules, a nonhelical region involving 10-15 amino acid residues per α chain exists at the N-terminal end continuous with the helical portion. It is in this N-terminal region that intramolecular crosslinking has been shown to occur [24,57].

III. FIBRILLAR FORMS DERIVABLE FROM DILUTE COLLAGEN SOLUTIONS

Various types of aggregates or fibrillar forms can be derived from dilute collagen solution. Their detailed structural differences observed with the electron microscope depend on the conditions by which they are precipitated. By appropriate manipulation of pH, ionic strength, and temperature or by adding certain nonspecific agents, such as ATP (adenosine triphosphate) or chondroitin sulfate, collagen may be precipitated in a number of different fibrous modifications[58]. They have substantially aided in the interpretation of fibrous collagen structure at the molecular level.

An early concept of the interconvertibility of the various precipitated forms is illustrated in Fig. 3. The fact that all the different ordered forms of precipitated collagen observed by electron microscopy staining techniques are associated with the apposition of polar regions of different molecules and that the mode of precipitation depends strongly on ionic strength, pH, and the presence of highly charged polyanions such as ATP,

suggested that the mode of precipitation is determined predominantly by electrostatic forces[59]. The nature of the polar groups of the collagen that play the dominant role has been intensively pursued. Martin et al[60] implicated the imidazole groups, a conclusion subsequently questioned by Bensusan et al[61] who, in turn, postulated an important role for the arginyl residues. Earlier work of Bensusan and Scanu[62] indicated that ionization of the tyrosyl residues was an important step in native-fibril formation. Kahn et al.[63] have suggested a specific rôle for the inorganic ions in the aggregation media through ion binding with its consequent alteration of surface-charge pattern.

It has been clear since the work of Gross, Highberger and Schmitt[21,58] that the electron-microscopically observed, polarized-band pattern of the segment-long-spacing (SLS), precipitated-form of soluble collagen represents a molecular map or "finger-print" of the distribution of both polar and non-polar side chains along the length of the rod-like collagen molecule. Hodge and Schmitt[64] reasoned that in principle the band patterns of other aggregation states should be obtainable by multiple photographic exposure of the SLS pattern with appropriate longitudinal displacements and with due regard for centrosymmetry, or the lack of it. In the case of the native-type fibril, an optical synthesis resulted in a band pattern in qualitative agreement with the observed native-type pattern when the successive displacement approximated 1/4 of the length of the collagen molecule as depicted by the SLS precipitated form. Additional evidence in support of the "quarter stagger" array in native fibrils was provided by experiments in which SLS crystallites were grown under appropriate conditions on native-type collagen fibrils (Fig. 4). The "obviousness" of the continuity of bands in these "dimorphic" aggregates claimed by Hodge and Schmitt[64]

has more recently been questioned[65].

The original "quarter stagger" concept of the native collagen fibril which was based on positive-staining techniques required modification with the introduction of evidence derived with negative-staining methods. Negative-staining techniques showed that the length of the SLS unit as determined by positive-staining was in fact underestimated by 10 percent. Olsen[66,67] after analysis of electron micrographs from negatively stained preparations postulated that the mechanism of linear polymerization of the collagen molecules to form protofibrils was by an overlapping of the terminal 10 percent of the molecule. Hodge and Petruska[52] came to a similar conclusion, and in 1965 Hodge, Petruska and Bailey[68] altered the original concept of "quarter stagger" packing by postulating that the "protofibril has an intrinsic periodicity of 4 D", where D refers to the repeat period within which is observed a major light and a major dark band on electron microscope examination of negatively-stained collagen. Petruska and Hodge[69] had determined the length of the collagen molecule by a negative contrast technique to be 4.4D. On close examination of the band pattern of the fibrous form of SLS (F-SLS) generated by dialysing an acid solution of collagen against water prior to the addition of ATP, it was observed that all the D distances, including that across the junction between longitudinally adjacent molecules of the protofibril, were equal to 1.0 D[52]. From this result and the value of 4.4 D for single SLS segments, they postulated the formation of protofibrils must involve a specific end-to-end overlap of 0.4 D, i.e., about 10 percent of the molecular length. The same degree of overlap was presumed to be present in native-type fibrils. It was further postulated[68] that in the hydrated structure of native-type fibrils, there must be holes or pores about 0.6 D (400 A) in length with an effective cross-sectional diameter about that of the

collagen molecule. A two-dimensional representation of the packing arrangement of collagen molecules envisioned in the native-type fibril is shown in Fig. 5. Similar adjustments in the mode of packing of FLS structures from that shown in Fig. 3 have been postulated by the same authors[68].

While the proposed overlap explanation by Hodge and Petruska of the packing of collagen molecules in a native fibril very plausibly explains the cross-striation pattern seen in a two-dimensional pattern, the transition to a three-dimensional arrangement which maintains a quarter-stagger relationship between all nearest neighbor monomer units has been difficult to perceive. Smith[70] deduced that only 2/3 of monomer contacts in the native structure could be in quarter-stagger agreement.

Cox, Grant and Horne[65] have been critical of the central role assigned in the modified "quarter stagger" theory to the formation of a long linear protofibril, and they have questioned whether such protofibrils as depicted in the overlap hypothesis could be packed by a process of "quarter stagger" to form native-collagen fibers. They have proposed a mechanism of aggregation in which the formation of long linear protofibrils becomes unimportant. They postulate a collagen molecule that can be divided longitudinally into 5 main bonding zones (0.4 D)each) separated by 4 non-bonding zones (0.6 D each) (Fig. 6) . The bonding zones are thought to contain amino acids arranged in a mutually specific manner so that when two such bonding zones approach one another closely in proper alignment, a strong lateral attraction results from the formation of many intermolecular electrostatic and hydrogen bonds. The structurally complementary bonding groups are distributed around the molecule in bonding zones giving the three-dimensional result. The absence of a detectable ordered electron microscopic pattern in early stages of native fibril formation suggested

to these authors that the process was fundamentally random. It was postulated that native-collagen fibrils with 640 Å periodicity (D) were formed by allowing a main bonding zone on one collagen molecule an equal probability of initially cross-bonding in a structurally complementary manner with any of the five main bonding zones on a different macromolecule. The combining molecules are oriented in the same direction. This method of aggregation enables a fibril to be formed by repetition of a single process and permits individual collagen molecules or aggregates of such molecules to be added to a growing fibril.

Doubt has been expressed that a theory of essentially random accretion can in fact account for the formation of the native fibril *in vivo*[71,72]. Bard and Chapman[71] prefer a mechanism involving very specific interactions precisely located on the collagen molecule. Alterations in the environment lead to a loosening of this specificity with the subsequent formation of structures which show abnormal properties of growth and aggregation.

Veis et al[72] have objected to representations which treat the collagen monomer as a rigid rod with radial symmetry of its properties at any particular point along the rod. They point to the fact that while two of the component polypeptide chains of the collagen molecule ($\alpha 1$) are essentially identical, the third peptide strand ($\alpha 2$) has a different composition. To achieve fibril stability through a packing arrangement which maximizes electrostatic interactions between rods, Veis et al[72] propose that a rotational phasing perpendicular to the collagen molecular long axis must exist in addition to the axial quarter-stagger. Evidence to support this hypothesis is drawn from earlier work which showed (1) that no molecular weights intermediate between the γ components (3×10^5) and the δ components (1.2×10^6) could be detected

among the intermolecular polymers derivable from denatured collagen[73], and (2) that addition of salt-free ATP to γ_{111} solutions, renatured by cooling to 4°C, produced copious amounts of monomeric SLS whereas the same procedure when applied to γ_{222} produced fibrous precipitates with native type periodicity. They concluded that while α1 chains can be aligned and intermolecularly crosslinked in vivo with their lengths in register, α2 chains are aligned and intermolecularly crosslinked in the quarter-stagger array. The aggregated structure proposed by Veis et al [72] for the native fibril then is a right-handed helical arrangement of four collagen molecules displaced successively from the origin by 0, 1, 2 and 3 fundamental repeat distances, D, along the axis and 90° about the axis. A second tetramer of the rod-like molecules fits directly into the first tetramer, leaving a 0.6 D unit hole and a 0.4 D overlap, and the process may continue to make microfibrils of indefinite length in which each monomer is in quarter stagger with respect to its neighbors. The model in the view of those proposing it is in accord with observed physicochemical behavior of native collagen fibers.

IV. KINETICS OF COLLAGEN PRECIPITATION

In earlier sections, collagen molecules were described as existing at low temperatures in dilute solutions as highly asymmetric, internally order structures. Dilute solutions of such molecules are known to be stable. However, because of the space-filling problem that is encountered with highly asymmetric macromolecules of any type, isotropic solutions can only exist at extremely high dilution[74-76]. As the solute concentration is increased, phase separation must occur as a consequence of the asymmetry of the large collagen molecules. In the absence of any intermolecular interactions, both phases that result are predicted to be dilute with one being isotropic and the other

anisotropic. However, supplementation of the consequence of high asymmetry with a comparatively low interaction energy is predicted to cause the formation of a concentrated phase with the near depletion of solute from the dilute phase. The change from a dilute isotropic solution to a dense ordered phase should occur reasonably abruptly and possess the general characteristics associated with a phase transition. It was therefore logical to see if experimental observations of the precipitation of native-type collagen fibrils were indeed consistent with the general theory outlined above.

A. Fibril Formation as a Nucleation and Growth Process

Precipitation of native-type collagen fibrils can be studied isothermally[77-84] or by raising the temperature at some selected rate[85,86]. Early isothermal studies[78-80] revealed that the rate of fibril formation (determined by measuring the turbidity of collagen solutions) was dependent on pH, ionic strength, and temperature and could be sensitive to the presence of specific ions in the aggregating media. Wood and Keech[83] in an electron microscope study examined the distributions in fibril diameter produced in native-type fibrils which were precipitated isothermally. These fibrils were formed in pH 6-8 buffer solutions of collagen at selected temperatures in the range 21-37°C. Large fibrils and a greater range in diameter were favored by precipitation at low temperature and pH, but high ionic strength at neutral pH also promoted formation of large fibrils.

Isothermal studies demonstrated that native-type collagen fibril formation took place in two steps, i.e., a lag or induction period, followed by a period of rapidly increased precipitation. Such kinetics indicated that nucleation and growth processes were involved. Nucleation processes possess certain unique features which distinguish them from the usual chemical and physical

processes. Cassel et al[84] reasoned that verification of a nucleation act in the isothermal precipitation of native fibrils would indeed permit the conclusion that a phase change was involved and would allow the formal physical chemical classification of the phenomenon along the lines previously discussed.

Mathematical analyses of the rate of fibril formation were given by Wood[80] and by Cassel et al[84]. Wood, assuming that reaction of the soluble collagen particles with the surface of growing fibrils controlled the rate of growth and employing the nucleation-growth concept, as applied by Waugh[87] to the precipitation of insulin fibers, derived equations which qualitatively accounted for (1) the occurrence of a lag period in precipitation during which nucleation predominates over growth; (2) the time course of precipitation after the lag period, and (3) the observation that the final distribution of fibril width is determined during the lag period.

Cassel et al[84] observed the kinetics of native-type fibril formation over a wide range of collagen concentrations (0.001-0.40 percent) at temperatures of 16-40°C. The magnitude of the very strong, positive temperature-coefficient of the precipitation was noted as not being reconcilable with that associated with normal chemical reactions. A dependence of the rate of precipitation on concentration, while not nearly as dramatic as that of temperature, was demonstrated, the more concentrated systems precipitating at a more rapid rate. Defining the relative extent of the precipitation X as

$$X = \frac{C_o - C}{C_o - C_s}$$

where C_o is the initial collagen concentration, C the concentration of collagen in the supernatant at the time t, and C_s the corresponding quantity at the

termination of the process, it had been shown earlier[77] that

$$X = E/E\alpha$$

where E is the extinction at time t and $E\alpha$ the corresponding quantity upon completion of the precipitation. Defining a "half-time" for the process, $t_{1/2}$, as the time required for half the maximum value of the extinction coefficient to be achieved, the rates of precipitation were quantitatively compared in plots such as that shown in Fig. 7. The logarithmic plot is necessitated by the broad time scale encompassed by the measurements. The shapes of these time-temperature curves are quite similar for each concentration and if re-scaled, utilizing a fictitious temperature for each concentration, a single curve will result.

Plots of the extent of precipitation, X, as a function of time, t, were made, a typical one being that of Fig. 8. As illustrated in the right-hand box of the figure, at temperatures above 16°C, the curves are superposable to X=1 merely by shifting each an appropriate distance along the horizontal axis. This superposability permitted the conclusion that the kinetics of the process were indeed explicable by a reduced variable involving time and temperature.

According to nucleation theory for precipitation[88-90], the rate of nucleation can be expressed by

$$N = N_o \exp\left[\frac{-b\sigma^3}{RT(\Delta F_A)^2}\right]$$

where N_o is a term dependent on the concentration of solute molecules and the activation energy for the growth of a nucleus, b is a geometrical factor describing the shape of the nucleus, σ is the interfacial energy per unit area

between a nucleus and the mother liquor, and ΔF_A is the free energy involved in the transfer of a participating molecule between two macroscopic phases. At constant temperature, ΔF_A will be proportional to the logarithm of the supersaturation ratio, and at constant composition, there will be proportionality with the difference in the temperature from that at equilibrium. In effect, the term $\sigma^3/(\Delta F_A)^2$ is related to the size or the number of molecules required to form a nucleus of minimum-sized stability. This quantity is very sensitive to temperature and supersaturation ratio, and consequently the rate of nucleation is affected.

For a dilute system of nucleating particles, growth will be governed by processes occurring either at the particle-solution interface or by the diffusion of the solute component to the interface. Cassel et al[84] treated these processes independently in the analysis of their data. The functional dependence of the extent of collagen fibril precipitation on a reduced time variable, κ, as deduced from equations derived for diffusion - and interface - controlled reactions is shown in Fig. 9. For the range 18-30°C, good agreement with interface control (assuming a fixed number of particles) is obtained for $X \geq 0.2$ in accord with the conclusion of Wood[80]. The data at 16°C, is, however, more adequately described by the equation for diffusion-controlled growth. Both Wood[80] and Cassel et al[84] were in agreement in finding that nuclei are not constant in number during the lag period, but instead increase with time. Likewise, the results of Wood[80] and of Cassel et al[84] clearly substantiated the concept that a phase transition is involved during the isothermal precipitation of native-type collagen fibrils, commonly referred to as "heat precipitation".

B. Role of Polyanions in Collagen Precipitation

It has been indicated several times that since soluble collagen can be reconstituted to native-type fibrils in the absence of added polyanions, mucopolysaccharides are unlikely to play any direct role in the in vivo fibril forming process[78,91,92]. Lowther[93] suggested an indirect role through the formation of an extracellular viscous gel which may play the role of confining collagen molecules near a cell surface and thus by a concentration effect assist in their aggregation to fibrils.

It has been known for some time[94,95] that mucopolysaccharides cause precipitation of collagen from tissue extracts over a pH range 3-4. The probability that these precipitations were a demonstration of the non-specific co-precipitation of oppositely charged colloids was pointed out by Jackson and Bentley[96]. In many cases the fibrils precipitated under these circumstances were either devoid of structure or showed spacings other than the 640 Å spacing associated with native fibers[94].

The first observations of the effect of polyanions such as chondroition sulfate, hyaluronic acid, keratin sulfate, and heparin sulfate in a neutral pH range, indicated little or no effect on the rate of native-type collagen fibril formation. Wood[81], however, using the more discriminatory temperature of 25°C in a careful study of the effect of such polyanions, showed that very low concentrations of chondroitin sulfates A and C and keratosulfate accelerated the nucleation phase and rate of fibril development, whereas heparin, deoxyribonucleic acid, and a series of dextran sulfates of different molecular weights and degrees of sulfation had an opposing effect. Such effects were eliminated by prior dialysis of the collagen solution against phosphate buffer. On the

other hand, chondroitin sulfate B and hyaluronic acid were without effect on the fibril formation. The electron microscope observations of Keech[97] indicated that precipitation-accelerators produced thinner fibrils, whereas the presence of inhibitors provided fibrils that were thicker.

For compounds to have an accelerating effect on fibril formation, the structural requirements are apparently severe since compounds as closely related as chondroitin sulfates A and B have different effects. Evidence of similar sensitivity is provided by the results of Caygill[98] who observed that while ascorbic acid delays or, depending on concentration, prevents native-type fibril formation under conditions similar to those expected in vivo, dehydroascorbic acid is without affect. Trnavska et al[99], examining the effect of certain intermediary metabolites of the aldehyde and benzoquinone type on the kinetics of fibril formation at 30°C in neutralized, acid-soluble collagen preparations of rat skin, found homogentistic acid to be much more potent than gentistic acid in its ability to decrease the lag phase, i.e., in enhancing the nucleation process.

From measurements of the rate of turbidity developed at 25°C in neutralized, acid-soluble collagen extracts of skin, Németh-Csóka and Kaiser[100] concluded that the effect of chondroitin sulfate-A on fibril formation was dependent on the age of the rat used as the source of the collagen. Addition of chondroitin sulfate-A was found to reverse the order as regards the relative rate of fibril formation observed in neutralized, acid-soluble skin collagens derived from young, middle-aged (the most rapidly precipitating form in the control experiment), and old rats (the most rapidly precipitating form in the presence of chondroitin sulfate-A). While chondroitin sulfate-A significantly accelerated fibril formation in the "young" and "old" collagen, it had a

negligible effect - in fact, a slight inhibitory one on the "middle-aged" collagen. However, a factor of undetermined importance in these experiments is the presence of 20-30 percent of non-collagenous protein in these acid-soluble collagen preparations.

Toole and Lowther[101] have recently described precipitation of acid-soluble collagen by chondroitin sulfate-protein. At 40°C, instantaneous precipitation occurred at physiological pH and ionic strength involving approximately 2/3 of the total collagen, and electron microscope examination indicated that native-type fibrils were precipitated. Further addition of chondroitin sulfate-protein did not give added precipitate. Heating the supernatant at 37°C caused precipitation after a lag period dependent on the concentration of the components. The lag period observed in the fibril formation of the supernatant was four to five times that observed on a portion of the untreated solution (not containing chondroitin sulfate-protein) diluted to comparable concentration. These authors suggested that the original collagen solution contained two fractions differing both in ability to react with chondroitin sulfate-protein and to form fibrils at 37°C. Toole and Lowther's[101] schematic representation of the interactions between components of a collagen solution and chondroitin sulfate-protein are shown in Fig. 10.

V. SOME THERMODYNAMIC ASPECTS OF COLLAGEN AGGREGATION

A. Phase Diagram Determinations

In collagen precipitation the free energy of forming a critical-size nucleus will be dependent on the undercooling and supersaturation, and the steady-state rate of nucleation will be similarly affected[84]. It is important, therefore, that the equilibrium phase-line, or solubility curve, be established, because it is from this curve that nucleation rates must be

reckoned. A schematic representation of the transitions and phase equilibria involving collagen molecules is given in Fig. 11. The aggregation process, which is the subject of this review, involves transition from phase II to phase III. In Fig. 11, the boundary between these phases is shown by a solid line. In agreement with the positive temperature coefficient of the aggregation process (Fig. 7), this line has a negative slope rather than the positive slope of the dashed line. (Transitions to phase I represent denaturation processes and are not of concern in the present review.) It is to be noted that an isotropic solution of rod-like collagen molecules can be converted directly by appropriate temperature increase into either an ordered anisotropic phase or a disordered phase containing denatured molecules. Conversion into the latter phase may also occur via the anisotropic phase with sufficient increase in temperature. A specific phase diagram would depend on the conditions of pH, ionic strength, and salt types present.

Pseudo-phase diagrams with temperature and salt concentration as variables have been determined by Bianchi et al[85,86]. From their work, a systematic overview of the effects of salt concentration, salt type and pH on transformation temperatures can be obtained. Freshly prepared rat-tail-tendon-collagen solutions of a given pH and salt concentration were equilibrated in the cold for at least 12 hours after which the temperature was gradually increased. From visual observation and viscosity measurements, the nature of the physical state of the collagen molecules under a specific set of conditions was determined, and their data, plotted as pseudo-phase diagrams, are given in Fig. 12. The authors concluded that the variety of effects investigated could be most usefully rationalized by the assumption of one primary mechanism of interaction between salts and protein, namely a binding of the ions with the collagen substrate.

The adsorption of cations was envisaged as increasing the stability of the helical form in isotropic solution relative to that in the ordered native-fibril form. Adsorption of anions produces the reverse stability relationship.

B. Hydrophobic Bonding Aspects

Hydrophobic bonding is the term used to describe the tendency of non-polar groups to adhere to one another in an aqueous environment. The aversion of the apolar side chains of globular proteins for water results in the tendency for these residues to be in the interior of these rather compact spherelike conformations, an arrangement responsible for the hydrophobic bond stabilization of these structures[102].

Evidence of a role for hydrophobic bonding in native-type collagen fibril formation has been presented by Cassel[103] who determined the temperature coefficients of the proportions of collagen aggregated in the three processes: native-type fibril formation as induced in the heat precipitation process, ATP-induced lateral aggregation giving the SLS form, and the end-to-end aggregation that occurs on dialysis of weakly acidified, dilute collagen solutions against distilled water[64]. The endothermicity of the native-type fibril formation was in strong contrast to the exothermic character of each of the other aggregation processes.

The opposing effects of temperature along with the contrasting effect of ionic strength on the aggregations were interpreted by Cassel[103] as evidence against any interpretation that the forces governing the native-type fibril formation are a simple composite of those directing the other two aggregations. Despite the role of electrostatic forces in the highly specific alignment of collagen units in the native aggregate, it was concluded that the greatest stabilizing contribution was that achieved through entropy-driven hydrophobic

bonding. The thermodynamics of this type of bond have been discussed by Kauzmann[102] in some detail. Cassel[103] and Bianchi et al[85] independently postulated that the required entropy gain in transferring collagen molecules from the isotropic phase to the ordered anisotropic phase of native-type fibrils was provided by a disruption of water structure around the individual rod-like collagen molecules. The non-helical end regions of the collagen molecules were not considered to play a dominant role in this aggregation although intermolecular cross-links involving these regions could presumably be responsible for diminished reversibility of the aggregation noted with time.

Supporting the interpretation that disruption of water structure plays an important role in native-type fibril precipitation is the fact that the energy of activation for this process decreases with increased addition of various alcohols known to act as breakers of water structure[104]. Cassel and Christensen[105] reasoned that an aggregation process placing apolar side chain groups in contact would diminish the organized water structure around such surfaces and should, in accordance with measurements made on hydrocarbon-water systems[102], be accompanied by a net increase in volume. By precise dilatometric measurement, they observed a volume increase of 0.8×10^{-3} ml/g of collagen in a system aggregating at neutral pH in phosphate buffer.

Fessler[106] concluded from apparent density measurements that nearly 1/4 of the water "shell" is lost on formation of native-type fibril, e.g., the water decreases from 19.0 to 14.5 m mole/g collagen. Cassel and Christensen[105] estimated that agreement between this calculated "water loss" and their dilatometrically measured volume increase required a density 1% greater than normal in the water involved in the quasi-crystalline water sheath, and hence concluded that the apparent density increase observed by Fessler was compatible

with their finding of a volume increase.

It should be noted that other polymeric substances, e.g., polyproline[107-110] and tobacco mosaic virus protein[111], exhibit aggregation phenomena very closely related to that of collagen in that these aggregations occur without change of polymer conformation and are favored by increasing temperatures. The active role of solvent water has been implicated in these phenomena as well.

C. Time-Dependent Changes in the Reversibility of Native-Type Fibril Formation

Judging from x-ray diffraction and electron microscope evidence, the native-type fibrils precipitated *in vitro* from collagen solutions are very similar to the collagenous material observed in various tissues. However, with the exception of rat-tail tendon, most collagenous tissue of mature animals yields little, extractable, undenatured collagen without the aid of enzymatic treatment.

The extent to which *in vitro*, heat-precipitated, collagen can be resolubilized by decreasing the temperature has been shown to depend on (1) the mode of collagen preparation, i.e., the resolubilization of acid-soluble collagen preparations after heat precipitation is apparently much less readily achieved than with neutral-salt soluble collagen precipitated in a similar manner[82]; the ionic strength employed during the heat precipitation[33]; (3) the relative extent to which a particular heat precipitation has been allowed to proceed[33]; and (4) the time lapse between complete precipitation and attempts at resolubilization[33,41,112].

Fessler[41] observed that while one fraction of neutral-salt-soluble rabbit-skin collagen was capable of repeated heat precipitation and re-solution

by cooling, a second fraction was relatively insoluble when subjected to similar temperature manipulation. Since the reversibly precipitated fibrils remained so after removal of the insoluble material from the system, Fessler concluded the heterogeneity was a property of the native, neutral, collagen solution and was not caused by the heat precipitation step. Of the two fractions, the one showing irreversible fiber formation was found by ultracentrifugation to have a significantly greater portion of β component, the formation of which involves crosslinking of any two of the α chains[82].

Gross[33] had suggested that the decrease in solubility of heat-precipitated collagen with time was due to a gradual attainment of a more ordered arrangement and closer packing of the collagen molecules in the precipitated fibrils under the influence of thermal motion. This reordering together with elimination of water molecules was presumed to produce stronger intermolecular attractions. Gross subsequently[113] specified that among these stronger intermolecular attractions were those which resulted in covalent crosslink formation. Supporting evidence for the occurrence of intermolecular crosslinking between collagen molecules resulting from the close proximity achieved in the heat precipitation step, was provided by Bannister[114]. Salt-soluble, rat-skin collagen, precipitated from solution at neutral pH and 37°C, partly resolubilized in 24 hours at 4°C. After denaturing the collagen components of the dissolved and nondissolved phases, analysis of the aggregated forms was performed by starch-gel electrophoresis. Compared with the parent salt-soluble collagen, the material remaining insoluble after heat-precipitation and subsequent cooling had significantly more of a component of low mobility, shown previously[115] to be an aggregate of collagen molecules.

Studies with *in vivo* altered collagens have provided an insight into the mechanism by which insolubility of collagen fibrils is developed both *in vivo* and *in vitro*. Animals in which lathyrism has been produced have been of particular value. Lathyrism, commonly induced experimentally by feeding the reagent β-aminopropionitrile, is characterized by mesenchymal deformities, loss of tensile strength in collagenous tissues, and dramatic increase in extractability of collagen from such tissues[116]. The molecular dimensions, conformation and rate of fibril-forming ability of lathyritic collagen does not seem to be grossly altered[117] although there is evidence for reduced intramolecular crosslinking characteristics of the *in vivo* maturation process[35,118,119].

The fibrils precipitated at 37°C from neutral solutions of collagen derived from lathyritic animals do not develop the insolubility with time that is characteristic of collagen from normal animals (Fig. 13)[117]. The inability to develop insolubility in fibrils aged at 37°C was termed by Gross an "intermolecular defect". Subsequent investigators have correlated this defect with an aldehyde component of the collagen structure. The collagen of animals fed the lathyrogen β-aminopropionitrile apparently becomes extractable as a result of an inhibition of crosslinking that involves aldehydic forms derived *in vivo* from both lysine[120,121] and hydroxylysine[122-124]. Blockage of crosslinking by lathyrogens is presumed to involve inhibition of the enzymatic oxidative deamination step in the conversion of lysyl residues to allysyl (α-aminoadipic-δ-semialdehyde). Penicillamine feeding on the other hand prevents utilization of these allysine residues in a subsequent step of the crosslinking procedure[125,126]. When crosslinking of two α chains occurs within the collagen molecule, i.e., intramolecular crosslinking, the β-components that are formed can be readily characterized since they are soluble following denaturation of the collagen

and are separable from α chains on the basis of size and charge. However, when crosslinks are formed intermolecularly little of the collagen dissolves even under denaturing conditions, the products formed by such crosslinking are not readily characterized and hence the specific crosslinking mechanism is difficult to determine. It is generally assumed, however, that reactions similar to those involved in the intramolecular crosslinking occur. While there is strong evidence that intramolecular crosslinking occurs in the non-helical N terminal regions of the collagen molecule, intermolecular cross-linking is presumably not so restricted.

Tanzer et al[127] in a study of the role of aldehydes in crosslinking, investigated the characteristics of a product produced by reaction of thio-semicarbazide (TSC) with embryonic calf skin collagen. Two moles of TSC were found to react per mole of collagen. The reaction was without effect on the intramolecular crosslinking structure as judged by carboxymethylcellulose chromatography and acrylamide gel electrophoresis of the thermally denatured TSC-collagen. While the rate of aggregation to fibrils at neutral pH and 37°C was unchanged from that of the control by the TSC treatment, the stability of the precipitated fibrils to a temperature of 5°C was markedly diminished. Electron microscopic examination of the TSC-collagen fibrils revealed the normal native-type pattern. The exact nature of the bond between TSC and collagen could not be determined, but on the basis of absorption spectra, it was suggested that thiosemicarbazones were formed by reaction with aldehydic carbonyl groups in collagen. The two moles of "aldehyde" per collagen molecule would provide one more than the minimum number required to form a continuous, covalently linked polymeric network between adjacent collagen molecules. The mechanism for intermolecular crosslinking was envisaged as a condensation of aldehyde and

ε-amino group of either lysine or hydroxylysine residues to give a Schiff base. Support for this mechanism was provided in a subsequent paper by Tanzer[128] who found that treatment of precipitated collagen fibrils with sodium borohydride, a procedure designed to reduce Schiff bases, produced a firmly cross-linked species as determined by solubility, thermal shrinkage and dissolution, as well as by examination of the denatured protein by ultracentrifugation, gel filtration, polyacrylamide electrophoresis and carboxymethyl cellulose chromatography.

Deshmukh and Nimui[112] demonstrated a correlation between thermally-fractionated, neutral-salt-soluble collagen and the corresponding aldehyde content. The concentration of aldehyde present in the neutral-salt soluble collagen before precipitation, as well as that found in the different fractions isolated by repeated thermal precipitation and cold dissolution, is shown in Table 1. The aldehyde concentration of the collagen remaining in the supernatant decreased after every precipitation. After the fourth precipitation, the aldehyde content of the collagen is approaching that observed in collagens extracted from the tissue of animals fed lathyrogens such as β-aminopropionitrile. Table 1 also demonstrates that the portion of collagen returning to solution after the gel-forming incubation period is enriched in β components. The change in α/β subunit ratio from approximately 4 to 1.8 is considerably greater than the approximately 5 to 4 ratio as detected after a similar incubation period by Wood[82] who employed ultracentrifugation as the analytical tool.

VI. COLLAGEN AGGREGATION IN VIVO

A number of <u>in vivo</u> studies of fine structure have indicated that collagen precursors are secreted by the fibroblast[129]. Histological and electron microscopic examinations of tissue sections have shown that although the fibers

lie close to the cell surface, they are entirely outside the cell[130]. The extracellular aggregation hypothesis is strongly supported by the experiments of Fitton Jackson and Smith[131] and Kuwabara[132] who found in tissue cultures that during a period of active growth a soluble collagen was formed and secreted into the medium without the concurrent formation of collagen fibers. In older cultures on the other hand, collagen fibers appeared without any further increase in the total collagen content.

That the collagen molecule as defined in an earlier section (II B) is the in vivo precursor of collagen fibers is largely deduced from turnover studies using isotopically labeled amino acids. Jackson and Bentley[44] injected C^{14}-glycine into guinea pigs and at various time intervals determined the specific activity of collagen extracted by successive increments in ionic strength at neutral pH. The most highly labeled fraction was initially extracted by 0.14 M NaCl, but within twenty-four hours the peak of the activity was obtained from fractions which could only be extracted with 0.45 M NaCl and 1.0 M NaCl. From these observations, these authors proposed the following hypothesis for collagen fiber formation. Collagen fibrils are formed at the surface of the fibroblast. Further increase in size occurs by accretion from extracellular soluble collagen rather than by aggregation of fibrils. They propose two controlling processes (a) the formation of new fibrils and production of soluble collagen in the cell to be added to the fibrils, and (b) the increase of crosslinking activity with time. In this view, the deeper the collagen molecule in the fiber, the more firmly it will be crosslinked; and, conversely, the outer, loosely aggregated collagen will be more easily extractable.

The hypothesis of Jackson and Bentley[44] that growth of fibrils occurs by accretion to fibrils rather than by an aggregation of fibrils is in line

with the view that fibrogenesis is a nucleation and growth process[80]. The size of fibrils formed *in vivo* in a particular tissue is considered a function of the amount of collagen synthesized with equal importance given the number of fibrils formed. Fitton Jackson[133] found in an electron microscopic study of fibrogenesis in embryonic avian tendon that the diameter of fibrils in any one bundle of fibrils had a rather narrow distribution and concluded that they were formed almost simultaneously at a very early stage. Analysis of the development of the avian tendon showed that the diameter of the collagen fibrils increased steadily with age and further that the enlargement was linearly correlated with a reduction in the relative amount of inter-fibrillar material (a material distinct from the more ubiquitous ground substance) detected with each fibril. Extrapolation indicated an initial fiber diameter between 20-30 Å, a result interpreted as indicative of the presence of a "central core" presumed to act as a nucleus and deposited by one process with subsequent addition of other collagen molecules occurring by a different method. Veis et al[72] have proposed a similar central core. Their limiting microfibril model for the three-dimensional arrangement within collagen fibers consists of a helical arrangement of four collagen molecules displaced successively by a fundamental repeat distance D defined electron microscopically.

The *in vitro* studies of Wood[81] on fiber formation in the presence of chondroitin sulfate-A suggest that a high proportion of this substance will increase the rate of formation of nuclei and retard the subsequent rate of fiber growth. Therefore, secretion by the cell of a limited amount of chondroitin sulfate-A could produce thin uniform fibrils. It may be significant that cartillage with its high chondroitin sulfate-A content has thin fibrils, whereas skin with a high proportion of chondroitin sulfate-B, shown by Wood not to affect the nucleation step, has a much larger average fibril diameter.

In many studies[134-139], small extracellular fibrils have been observed in the early stages of *in vivo* collagen formation. There is little difficulty in detecting periodic banding in extracellular collagen fibrils greater than 100 Å in diameter. However, nonbanded fibrils approximately 100 Å in diameter or less are often also detected. Ross and Benditt[135] proposed that extracellular collagen fibrils 100 Å or less in diameter would not display the characteristic 650-700 Å periodicity associated with native collagen fibers. This suggestion was made on the basis of the dimensions of the collagen molecule and the possible ways in which these molecules could be packed together to form fibrils, assuming the quarter stagger, side to side arrangement of molecules in a linear array as proposed by Hodge and Schmitt[64].

The question of whether the collagen fiber generating system *in vivo* is in fact a self-limiting system has been the subject of debate[140]. Chapman[141] has observed that if the structure achieved by a packing of rod-like units in staggered array is to be intrinsically self-limiting in diameter, so that a fibril is formed, the rod-like units can only be quasi-equivalently related and some measure of strain energy must be stored in a unit or assembly. He proposed a design for self-limitation in which bond sites are arranged so that the rods twist slightly together; this requires both radial and axial shifts of the sites from the positions they would occupy in a straight-rod structure. The minimum-energy conformation of a large assembly of rods will then be a cylindro-helical structure with rod tilt increasing with radius. As more rods are added the tilt of the innermost rods decreases; the average strain energy per rod, and hence the energy barrier opposing the incorporation of new rods, increases toward an assymptotic limit with the presumption that radial growth

ceases when the energy barrier reaches a critical level. Chapman believes this model of the collagen fiber forming system more readily explains the observed mechanical and thermal behavior of collagenous tissue.

The extrinsic factors which affect the precipitation of collagen fibrils from solution *in vitro* include pH, ionic strength, temperature, concentration of collagen and the presence of various extraneous substances. Spontaneity of fibril formation has been demonstrated *in vitro*, but the molecular units used in these experiments of necessity have to be extracted from tissue. Indeed, whether these molecular units are in exactly the same state of molecular order as the cell's newly synthesized monomers destined to form fibrils *in vivo* has not been ascertained. Also unknown is whether, at the appropriate time in the sequence of events *in vivo*, the microenvironment of each fibril is altered to conditions similar to those which obtain when typical fibrils are formed *in vitro*.

USCOMM-NBS-DC

Table 4. *Aldehyde content and sub-unit composition of neutral-salt-soluble collagen and its various fractions separated during the process of repeated thermal precipitation and redissolution at 4°*

Neutral-salt-soluble collagen was incubated at 37° overnight, cooled at 4° and centrifuged. The procedure was repeated four times. All precipitates and supernatants at every step were analysed for their aldehyde content and sub-unit composition (α-sub-unit/β-sub-unit ratio).

No. of thermal precipitations	Total collagen (mg.)		Acetaldehyde content (μmoles/100 mg. of collagen)		Supernatant collagen α-sub-unit/β-sub-unit ratio
	Supernatant	Precipitate	Supernatant	Precipitate	
Original neutral-salt-soluble collagen	12·2	—	0·84	—	4·0
First precipitation	9·61	2·55	0·75	0·96	1·77
Second precipitation	8·53	1·14	0·71	0·77	1·81
Third precipitation	6·34	2·17	0·60	0·68	1·80
Fourth precipitation	4·58	1·66	0·53	0·59	1·82

REFERENCES

1. Zachariades, P. A., Compt. Rend. Soc. Biol., 52, 182, (1900).
2. Nageotte, J., Compt. Rend. Soc. Biol., 96, 172, (1927).
3. Nageotte, J., Compt. Rend. Soc. Biol., 96, 464, (1927).
4. Nageotte, J., Compt. Rend. Soc. Biol., 96, 828 (1927).
5. Nageotte, J., Compt. Rend. Soc. Biol., 97, 559, (1927).
6. Nageotte, J., Compt. Rend. Soc. Biol., 98, 15 (1928).
7. Nageotte, J., Compt. Rend. Soc. Biol., 104, 156 (1930).
8. Nageotte, J., Compt. Rend. Soc. Biol., 113, 841 (1933).
9. Nageotte, J. and L. Guyon, Compt. Rend. Soc. Biol. 113, 1398 (1933).
10. Nageotte, J. and L. Guyon, Compt. Rend. Ass. Anat., 29, 408 (1934).
11. Leplat, G., Compt. Rend. Soc. Biol., 112, 1256 (1933).
12. Leplat, G., Compt. Rend. Ass. Anat., 28, 404 (1933).
13. Faure-Fremiet, E., Compt. Rend. Soc. Biol., 113, 715 (1933).
14. Faure-Fremiet, E., Compt. Rend. Ass. Anat., 28, 277 (1933).
15. Guyon, L., Compt. Rend. Acad. Sci., 198, 975 (1934).
16. Wycoff, R.W.G., and R.B. Corey, Proc. Soc. Exptl. Biol. Med., 34, 285 (1936).
17. Hall, C. E., M. A. Jakus and F. O. Schmitt, J. Am. Chem. Soc., 64, 1234 (1942).
18. Schmitt, F. O., C. E. Hall and M. A. Jakus, J. Cellular Comp. Physiol., 20, 11 (1942).
19. Bear, R. S., Advances in Protein Chemistry, 7,69 (1952).
20. Orekhovich, V. N., 2nd Int. Congr. Biochem. Communications, p. 106 (1952).

21. Gross, J., Highberger, J. H., and Schmitt, F. O., Proc. Nat. Acad. Sci., U.S.A., 40, 679 (1954).

22. Boedtker, H., and P. Doty, J. Am. Chem. Soc., 77, 248 (1955).

23. Hall, C. E., Proc. Nat. Acad. Sci., U.S.A., 42, 801 (1956).

24. Piez, K. A. in "Treatise on Collagen I Chemistry of Collagen." (G. N. Ramachandran, ed.) P. 207 (1967), Academic Press, London and New York.

25. Harrington, W. F. and P. H. von Hipple, Advances in Protein Chemistry 16, 1 (1961).

26. Veis, A., "The Macromolecular Chemistry of Gelatin." Academic Press, New York (1964).

27. von Hipple, P. H. in "Treatise on Collagen I Chemistry of Collagen" (G. N. Ramachandran, ed.) p. 253 (1967) Academic Press, London and New York.

28. Ramachandran, G. N. in "Treatise on Collagen I Chemistry of Collagen" (G. N. Ramachandran, ed.) p. 103 (1967) Academic Press, London and New York.

29. Cowan, P. M. and S. McGavin, Nature, Lond. 176, 501 (1955).

30. Crick, F. H. C. and A. Rich, Nature, Lond. 176, 780 (1955).

31. Schnell, J. Arch. Biochem. Biophys. 127, 496 (1968).

32. Gross, J., J. H. Highberger and F. O. Schmitt, Proc. Nat. Acad. Sci., U.S.A. 41, 1 (1955).

33. Gross, J., J. Exptl. Medicine, 108, 215 (1958).

34. Piez, K. A., P. Bornstein, M. S. Lewis and G. R. Martin in "Structure and Function of Connective and Skeletal Tissue," (S. Fitton Jackson, R. D. Harkness, S. M. Partridge, and G. R. Tristram, eds.) p. 16 (1965), Butterworths, London.

35. Piez, K. A., M. S. Lewis, G. R. Martin and J. Gross, Biochim. Biophys. Acta, 53, 596 (1961).

36. Piez, K. A., E. A. Eigner and M. S. Lewis, Biochemistry 2, 58 (1963).

37. Engel, J. and G. Beier, Hoppe-Seyler's Z., Physiol. Chem., 334, 201 (1963).

38. Hodge, A. J., J. H. Highberger, G. G. J. Deffner and F. O. Schmitt, Proc. Nat. Acad. Sci., U.S.A., 46, 197 (1960).

39. Nagai, Y., J. Gross and K. A. Piez, Ann. N.Y. Acad. Sci. 121, 494 (1964)

40. Veis, A. and M. P. Drake, J. Biol. Chem. 238, 2003 (1963).

41. Fessler, J. H., Biochem. J. 76, 452 (1960).

42. Fessler, J. H., Biochem. J. 76, 463 (1960).

43. Candlish, J. K., Biochim. Biophys. Acta, 74, 275 (1963).

44. Jackson, D. S. and Bentley, J. P., J. Biophys, Biochem. Cytol. 7, 37 (1960).

45. Schmitt, F. O. in "Connective Tissue, Thrombosis and Atherosclerosis" (I. H. Page ed.) p. 43, (1959). Academic Press, New York.

46. Hodge, A. J. and F. O. Schmitt, Proc. Natl. Acad. Sci., U.S.A., 44, 411 (1958).

47. Schmitt, F. O., Federation Proceedings 23, 618 (1964).

48. Rubin, A. L., D. Pfahl, P. T. Speakman, P. F. Davison and F. O. Schmitt, Science 139, 37 (1963).

49. Drake, M. P., P. F. Davison, S. Bump and F. O. Schmitt, Biochemistry 5, 301 (1966).

50. Schmitt, F. O., J. Gross and J. H. Highberger, Exptl. Cell Research Suppl. 3, 329 (1955).

51. Hodge, A. J. and J. A. Petruska, in "Intern. Conf. Electron Microscopy, 5th" (S.S. Breese Jr. ed.) 1, Paper QQ 1 (1962). Academic Press, New York.

52. Hodge, A. J. and J. A. Petruska in "Aspects of Protein Structure" (G. N. Ramachandran ed.) p. 289 (1963). Academic Press, London.

53. Kühn, K., K. Hannig and H. Hörmann, Das Leder 12, 237 (1961).

54. Kühn, K., J. Kühn and K. Hannig, Hoppe-Seyler's Z. Physiol. Chem. 326, 50 (1961).

55. Hafter, R., and H. Hörman, Hoppe-Seyler's Z. physiol. Chem. 330, 169 (1963).

56. Hafter, R. Das Leder 15, 237 (1964).

57. Piez, K. A., Annual Review Biochemistry 37, 547 (1968).

58. Highberger, J. in "Chemistry and Technology of Leather" (F.O'Flaherty, W. Roddy and R. Lollar eds.) p. 65 (1956) Rheinhold, N.Y.

59. Highberger, J. H., J.Am.Leather Chemists' Assocn. 56, 422 (1961).

60. Martin, G. R., S. E. Mergenhagen and D. B. Scott, Biochim. Biophys. Acta 49, 245 (1961).

61. Bensusan, H. B., V. R. Mumaw and A.W. Scanu, Biochemistry 1, 215 (1962).

62. Bensusan, H. B. and A. W. Scanu, J.Am.Chem.Soc. 82, 4990 (1960).

63. Kahn, L.D., R.J. Carroll and L.P. Witnauer, Biochim. Biophys. Acta, 63, 243 (1962).

64. Hodge, A. J. and F. O. Schmitt, Proc. Natl. Acad. Sci., U.S.A. 46, 186 (1960).

65. Cox, W., R.A. Grant, and R. W. Horne, J. Royal Microscopic Soc. 87, 123 (1967).

66. Olsen, B.R.Z., Zellforsch 59, 184 (1963).

67. Olsen, B. R., Z. Zellforsch 59, 199 (1963).

68. Hodge, A. J., J. A. Petruska and A. J. Bailey in "Structure and Function of Connective Skeletal Tissue" (S. Fitton Jackson, R. D. Harkness, S. M. Partridge and G. R. Tristram eds.) p. 31 (1965) Butterworths, London.

69. Petruska, J. A. and A. J. Hodge, Proc. Nat. Acad. Sci., U.S.A. 51, 871 (1964).

70. Smith, J. W., Nature, Lond. 205, 356 (1965).

71. Bard, J. B. L. and J. A. Chapman, Nature, Lond. 219, 1279 (1968).

72. Veis, A., J. Anesey and S. Mussell, Nature, Lond. 215, 931 (1967).

73. Veis, A., and J. Anesey, J. Biol. Chem., 240, 3899 (1965).

74. Onsager, L., Ann. N. Y. Acad. Sci., 51, 627 (1949).

75. Isihara, A., J. Chem. Phys., 18, 446 (1950).

76. Flory, P. J., Proc. Royal Soc. (London), Ser. A, 234, 73 (1956).

77. Gross, J. Nature 181, 556 (1958).

78. Gross, J. and D. Kirk, J. Biol. Chem. 233, 355 (1958).

79. Bensusan, H. B. and B. L. Hoyt, J. Am. Chem. Soc. 80, 719 (1958).

80. Wood, G. C., Biochem. J., 75, 598 (1960).

81. Wood, G. C., Biochem. J. 75, 605 (1960).

82. Wood, G. C., Biochem. J. 84, 429 (1962).

83. Wood, G. C. and M. K. Keech, Biochem. J. 75, 588 (1960).

84. Cassel, J. M., L. Mandelkern and D. E. Roberts, J. Am. Leather Chemists Assoc., 57, 556 (1962).

85. Bianchi, E. C. Giuseppina and A. Ciferri, Biopolymers, 4, 957 (1966)

86. Bianchi, E., G. Conio, A. Ciferri, D. Puett and L. Rajagh, J. Biol. Chem. 242, 1361 (1967).

87. Waugh, D., J. Cellular Comp. Physiol. 49, Suppl. 1, 145 (1957).

88. Becker, R., Ann. Physik., 32, 128 (1938).

89. Turnbull, D. and J. C. Fisher, J. Chem. Phys., 17, 71 (1949).

90. Mandelkern, L., "Crystallization of Polymers," p. 215, (1964) McGraw-Hill Inc., New York.

91. Jackson, D. S., New England J. Med., 259, 814 (1958).

92. Gross, J. in, "Connective Tissue, Thrombosis and Atherosclerosis," (I.H. Page ed.) p. 77 (1959). Academic Press, New York.

93. Lowther, D. A. in, "Int. Rev. Connective Tissue Research," (D. A. Hall ed.) p. 63 (1963), Academic Press, New York.

94. Highberger, J. H., J. Gross, and F. O. Schmitt, Proc. Nat. Acad. Sc. U.S.A., 37, 286 (1951).

95. Randall, J. T., R. D. B. Fraser, S. F. Jackson, A. V. W. Martin and A. C. T. North, Nature (London), 169, 1029 (1952).

96. Jackson, D. S. and J. P. Bentley in, "Treatise on Collagen" (B. S. Gould ed.) 2, p. 189 (1968). Academic Press, New York.

97. Keech, M. K., J. Biophys. Biochem. Cytol., 9, 193 (1961).

98. Caygill, J., Biochim. Biophys. Acta., 181, 334 (1969).

99. Trnavska, Z., S. Sit'aj, M. Grmela and J. Malinsky Biochim. Biophys. Acta, 126, 373 (1966).

100. Németh-Csoka, M., and M. Kaiser, Acta morph. tomus 13, 119 (1965).

101. Toole, B. P. and D. A. Lowther, Biochem. J., 109, 857 (1968).

102. Kauzmann, W., Adv. Protein Chem., 14, 1 (1959).

103. Cassel, J. M., Biopolymers, 4, 989 (1966).

104. Bensusan, H. B., J. Am. Chem. Soc. 82, 4995 (1960).

105. Cassel, J. M. and R. G. Christensen, Biopolymers, 5, 431 (1967).

106. Fessler, J. H., in "Structure and Function of Connective and Skeletal Tissue" (G. R. Tristram, ed.) p. 80 (1966), Butterworths, London.

107. Mandelkern, L., J. Polymer Sci., 49, 125 (1961).

108. Mandelkern, L., "Crystallization of Polymers", p. 66 (1964), McGraw-Hill Inc., New York.

109. Blout, E. R. and G. D. Fasman, in "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.) p. 122 (1958), Pergammon Press, London.

110. Kurtz, J., A. Berger and E. Katchalsky, in "Recent Advances in Gelatin and Glue Research" (G. Stainsby, ed.) p. 131 (1958), Pergammon Press, London.

111. Ansewin, A. T., C. L. Stevens and M. A. Lauffer, Biochemistry, 3, 1512 (1964).

112. Deshmukh, K. and M. E. Nimni, Biochem. J., 112, 397 (1969).

113. Gross, J., Science, 143, 960 (1964).

114. Bannister, D. W., Biochem. J., 113, 419 (1969).

115. Hollmen, T. and E. Kulonen, Biochim. Biophys. Acta 93, 655 (1964).

116. Levine, C. I. and J. Gross, J. Exptl. Medicine, 110, 771 (1959).

117. Gross, J., Biochim. Biophys. Acta, 71, 250 (1963).

118. Martin, G. R., J. Gross, K. A. Piez and M. S. Lewis, Biochim. Biophys. Acta, 53, 599 (1961).

119. Schiffmann, E. and G. R. Martin, Arch. Biochem. Biophys., 138, 226 (1970).

120. Bornstein, P., A. H. Kang and K. A. Piez, Proc. Nat. Acad. Sci., U.S.A., 55, 417 (1966).

121. Bornstein, P. and K. A. Piez, Biochemistry, 5, 3803 (1966).

122. Bailey, A. J., L. J. Fowler and C. M Peach, Biochem. Biophys. Res. Commun., 35, 663 (1969).

123. Bailey, A. J. and C. M. Peach, Biochem. Biophys. Res. Commun., 33, 812 (1968).

124. Bailey, A. J. and L. J. Fowler, Biochem. Biophys. Res. Commun., 35, 672 (1969).

125. Nimni, M. E., J. Biol. Chem., 243, 1457 (1968).

126. Deshmukh, K. and M. E. Nimni, J. Biol. Chem., 244, 1787 (1969).

127. Tanzer, M. L., D. Monroe and J. Gross, Biochemistry, 5, 1919 (1966).

128. Tanzer, M. L., J. Biol. Chem., 243, 4045 (1968).

129. Ross, R., in "Treatise on Collagen" (B. S. Gould, ed.) p. 2, (1968), Academic Press, London and New York.

130. Porter, K. A. and G. D. Pappas, J. Biophys. Biochem. Cytol., 5, 153 (1959).

131. Fitton Jackson, S. and R. H. Smith, J. Biophys. Biochem. Cytol., 3, 897 (1957).

132. Kuwarbara, H. Japan J. Exptl. Med., 29, 627 (1959).

133. Fitton Jackson, S., Proc. Royal Society, B 144, 556 (1956).

134. Ross, M. H. in, "Proceedings of Fifth International Congress for Electron Microscopy" (S. S. Breese, Jr. ed.) p. T-13, (1962) Academic Press, New York.

135. Ross, R. and E. P. Benditt, J. Biophys. Biochem. Cytol. 11, 677 (1961).

136. Ross, R. and E. P. Benditt, J. Cell. Biol. 22, 365 (1964).

137. Chapman, J. A., J. Biophys. Biochem. Cytol 9, 639 (1961).

138. Karrer, H. E., J. Ultrastruct. Res., 4, 420 (1960).

139. Peach, R., G. Williams and J. A. Chapman, Am. J. Path., 38, 495 (1961).

140. Cohen, C. in, "Principles of Biomolecular Organization" (G.E.W. Wolstenholme and M. O'Connor eds.) p. 130 (1966), J. A. Churchill Ltd., London.

141. Chapman, J. in, "Principles of Biomolecular Organization" (G. E. W. Wolstenholme and M. O'Connor eds.) p. 129 (1966). J. A. Churchill Ltd., London.

Figure Legends

Fig. 1 Perspective view of the collagen structure for a height of about 30 Å. The α-carbon atoms along each of the three chains (A, B, C) of the triple helix are numbered. From Ramachandran[28] with permission.

Fig. 2 The projection of the prototype triple helical structure on a plane perpendicular to the fiber axis. The peptide units out of which the chains are built are shown as rods joining the α-carbon atoms. The numbers indicate the heights of the α-carbon atoms in Angstroms in the three chains A, B and C. Only hydrogens (marked H_1, H_2) can occur attached to the α-carbon atoms C_1, while β-carbon atoms in the L-configuration (marked C_β, C_β) can readily be attached to the α-carbon atoms C_2 and C_3. The five-membered ring of a proline residue is also shown attached to the atom C_3 in one of the chains (marked C_β, C_γ, C_δ, N). The carbon atoms are indicated by big circles and the hydrogens by small circles. From Ramachandran[28] with permission.

Fig. 3 Schematic representation of four of the modes of aggregation of monomeric collagen units. Native-type fibrils are formed by dialysis of an acid solution

of collagen vs. 1% NaCl. The SLS type precipitate is produced by addition of ATP to an acidified collagen solution. Addition of substances such as α-1 acid glycoprotein, chondroitin sulfate, hyaluronic acid or heparin to an acid solution of collagen followed by dialysis yields the FLS form. Dialysis of a dilute solution of collagen from a dilute acetic acid medium to distilled water generates the protofibrillar type aggregate shown in the right hand area of the figure. From Higberger[59] with permission.

Fig. 4 Dimorphic ordered aggregates of collagen produced by exposing native-type fibrils to a solution of collagen molecules and ATP at a pH favoring the formation of SLS. The Greek and English letters designate characteristic bands of the SLS and native-fibril forms respectively. From Hodge and Schmitt[64] with permission.

Fig. 5 A two-dimensional representation of the packing arrangement of collagen molecules in native-type fibrils. Longitudinal displacement of nearest neighbors by a distance D results in formation of a fibril of period D, with each period comprising an overlap zone of 0.4D and a hole zone of 0.6D. From Hodge et al[68] with permission.

Fig. 6 Diagram to illustrate the formation of a native-collagen fibril with 640 Å periodicity from collagen molecules of length 2800 Å. The collagen

molecule is divided into 5 main bonding or a zones (approximately 280 Å) separated by 4 main non-bonding or b zones (approximately 360 Å). Some of the bonding sites within the main bonding zones are represented by asymmetrically arranged white dots. By virtue of this asymmetry the collagen molecules are polarized. The white arrow heads and black dots at the ends of the molecules are inserted merely to emphasize this polarity. For the sake of clarity, minor bonding sites within the 4 main non-bonding zones are omitted.

When such collagen molecules are assembled so that a main bonding zone on one molecule is given an initial random choice in laterally cross-linking, in a structurally complementary manner, with a main bonding zone on another molecule the various stages depicted in the formation of the native fibril occur.

The morphological flexibility of the collagen molecules is emphasized and their ability to cross one another in both the A and B bands is illustrated. The ratio of the number of collagen molecules in the B band to the number in the adjacent A band approaches 4:5. From Cox et al[65] with permission.

Fig. 7 Semilog plots of half-times ($t_{\frac{1}{2}}$) vs. temperature for aggregating solutions of varying collagen concentration at constant ionic strength (0.5) and pH (7.5). (84).

Fig. 8 Left: Effect of temperature on extent of precipitation (X) at time t for 0.1% collagen solutions at constant ionic strength (0.5) and pH (7.5). Right: Superposition of isotherms to the isotherm at 20°C. (84).

Fig. 9 Fit of superposed isotherms of the extent of precipitation (X) against log time with calculated curves based on interface (continuous line in figure) or diffusion (dashed line in figure) controlled processes. Solid circles are experimental data obtained at 16°C. Open circles represent superposed isotherms (18°-30°C) of the type shown in Fig. 8. Graphs (a) and (b) are for 0.1% and 0.3% collagen solutions respectively. (84).

Fig. 10 Schematic representation of the interactions between components of a collagen solution and chondroitin sulfate-protein. Abbreviations TC_α and TC_β refer to "growth" and "nucleus-forming" collagen monomers respectively; TC_x refers to an aggregate of collagen; CSP is chondroitin sulfate protein. From Toole and Lowther[101] with permission.

Fig. 11 Schematic representation of the transitions and phase equilibria involving collagen molecules. Temperature and collagen concentration are the variables plotted.

Fig. 12 Pseudo phase diagrams for ternary water-collagen-salt solutions. The temperature of the transformations of the helical monomers (H) aggregates (C), helical monomers (H) randomly coiled forms (RC), and aggregate (C) to randomly coiled forms (RC) are plotted as a function of salt concentration (C_s). Collagen concentration is near 0.05%. From Bianchi et al[86] with permission.

Fig. 13 Opacity changes observed on heating and then cooling neutral solutions of collagen extracted from the skin of normal (———) and lathyritic (----) guinea pigs. Curves to left represent aggregation, those to the right dissolution. From Gross[117] with permission.

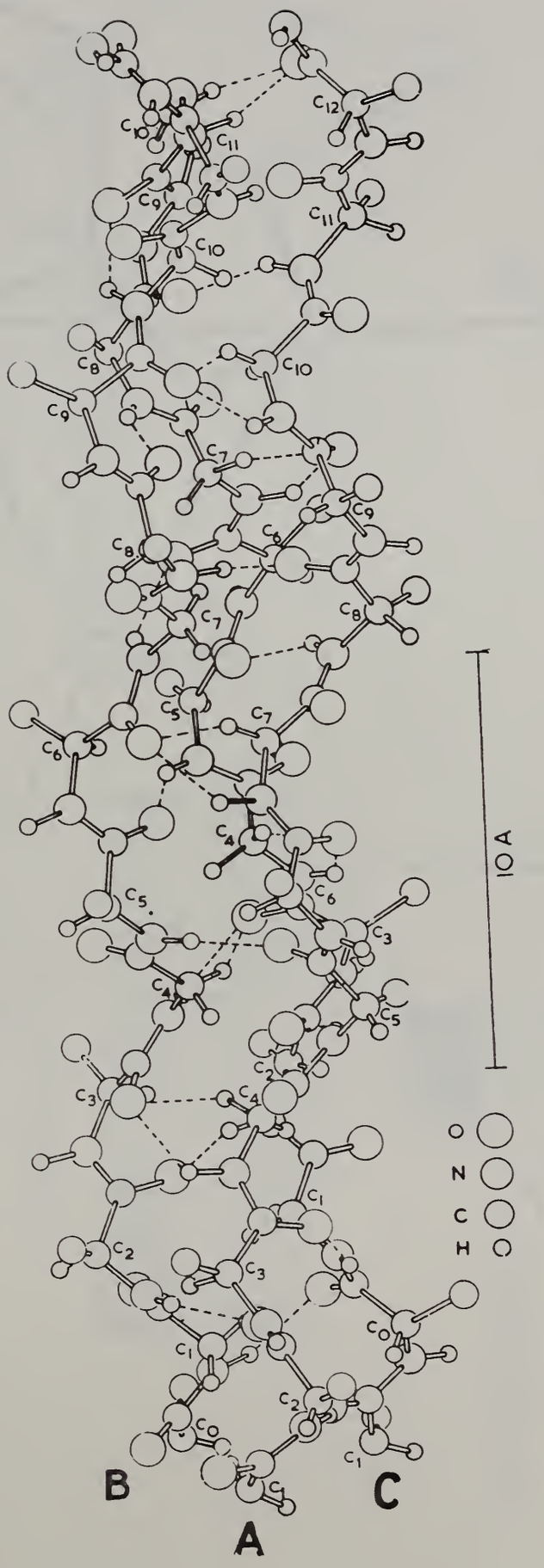
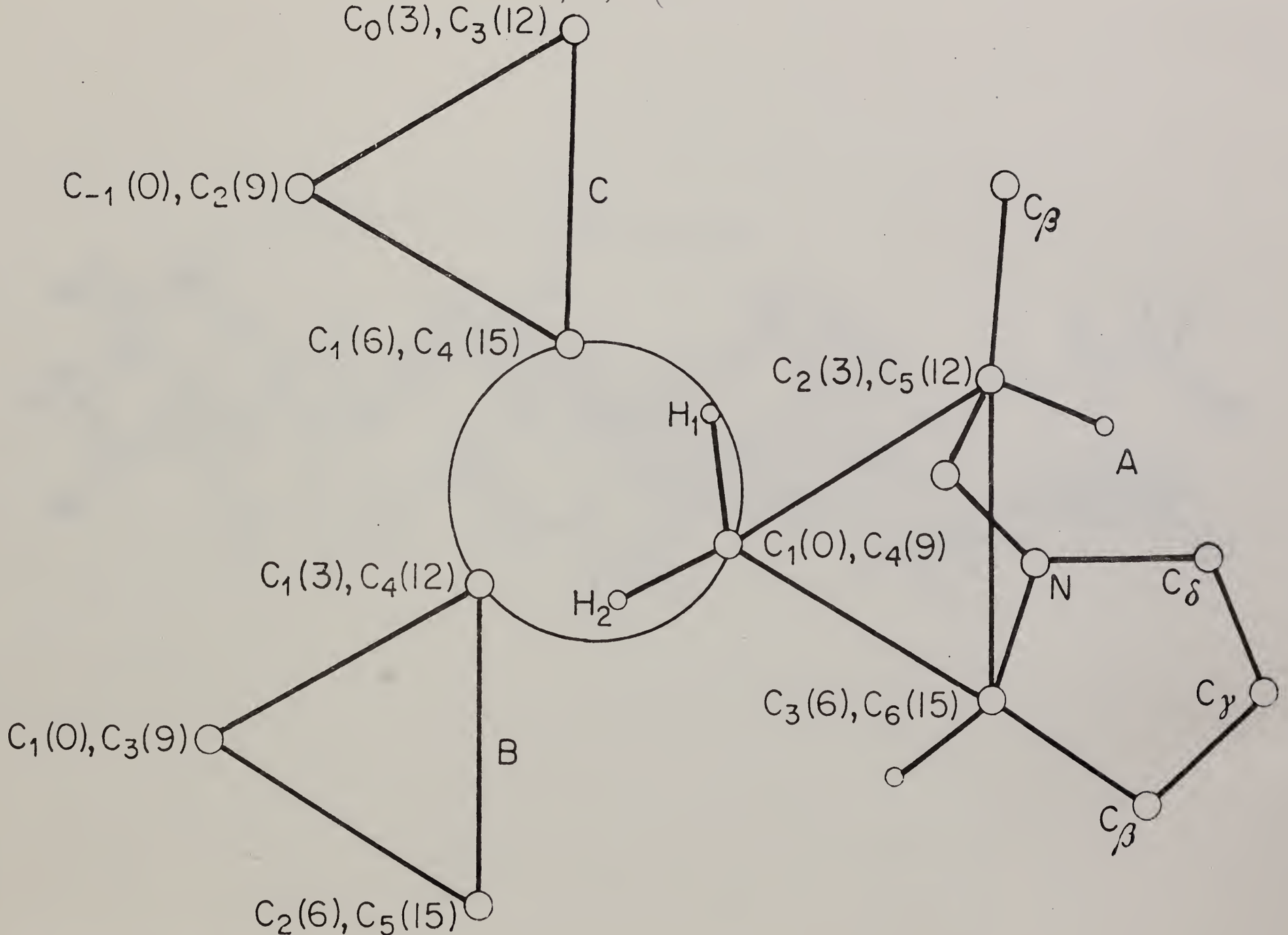


Fig 1

Fig 2


$C_0(3), C_3(12)$
$C_{-1}(0), C_2(9)$
C
$C_1(6), C_4(15)$
H_1
H_2
$C_1(3), C_4(12)$
$C_1(0), C_3(9)$
B
$C_2(6), C_5(15)$
$C_1(0), C_4(9)$
$C_2(3), C_5(12)$
C_β
A
N
C_δ
C_γ
$C_3(6), C_6(15)$
C_β

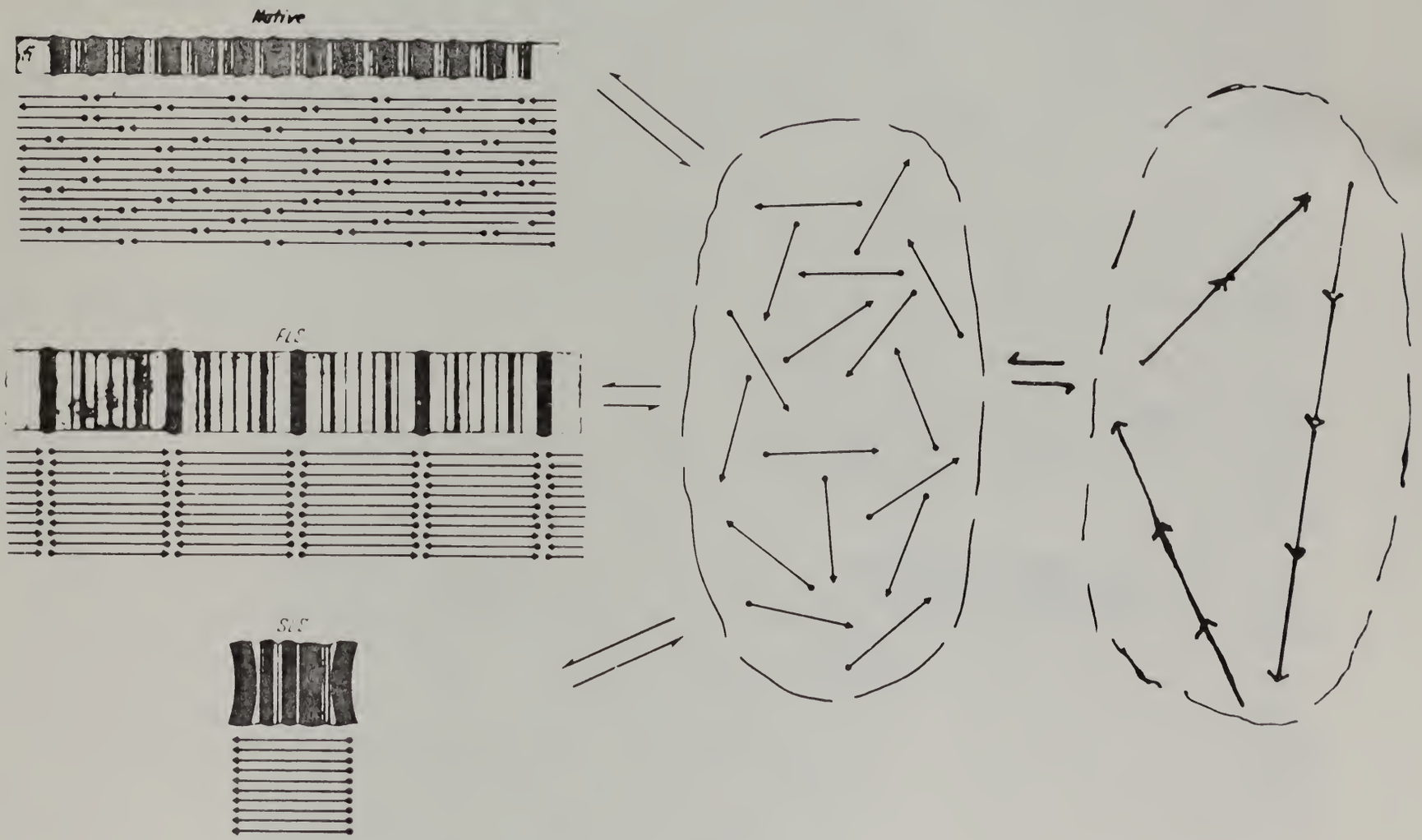


FIGURE 3. – Schematic representation of the modes of aggregation of the TC macromolecules in three structural forms, as produced reversibly from an acid solution of TC.

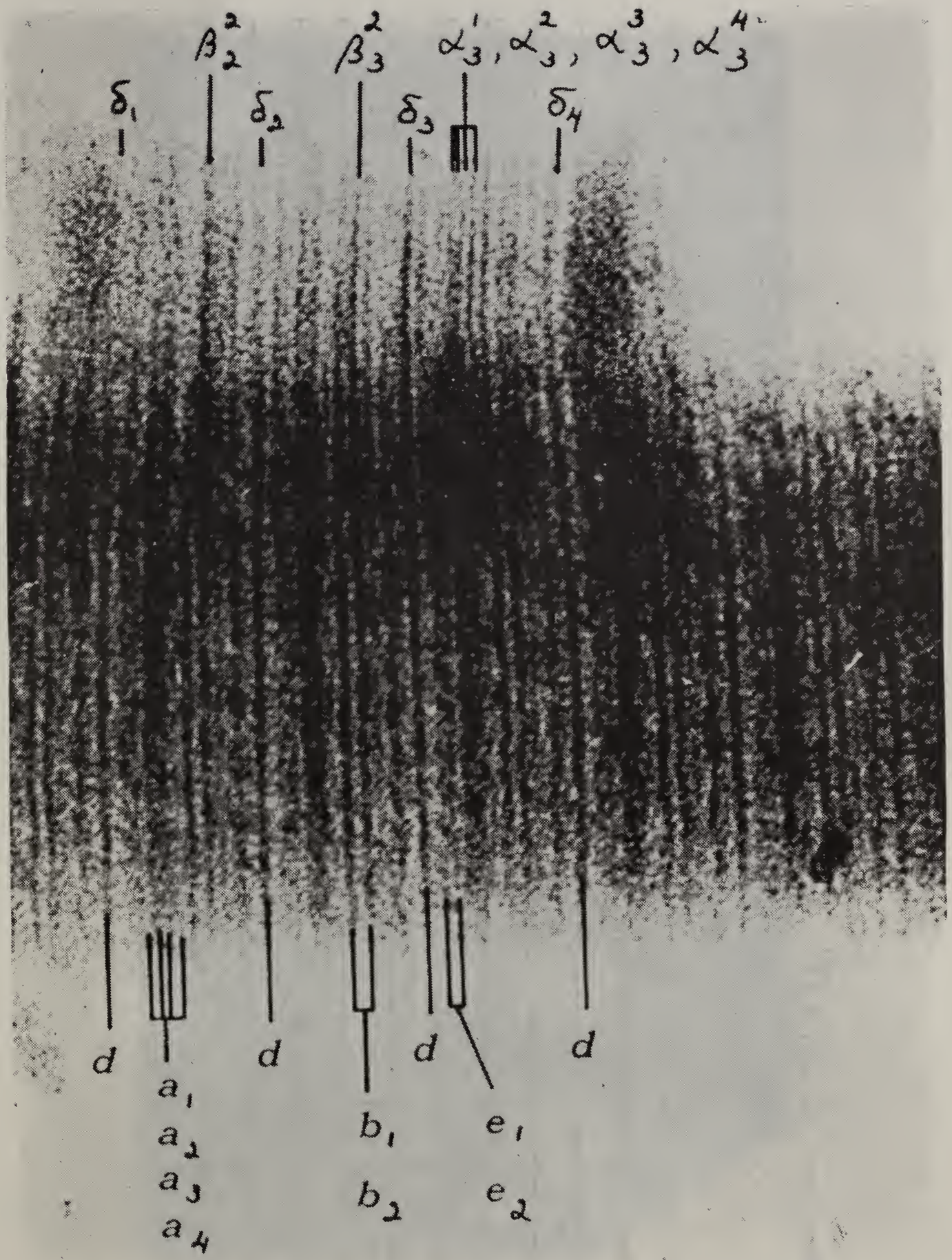

β_2^2
β_3^2
$\alpha_3^1, \alpha_3^2, \alpha_3^3, \alpha_3^4$
δ_1
δ_2
δ_3
δ_4
d
d
d
d
a_1
a_2
a_3
a_4
b_1
b_2
e_1
e_2

fig H

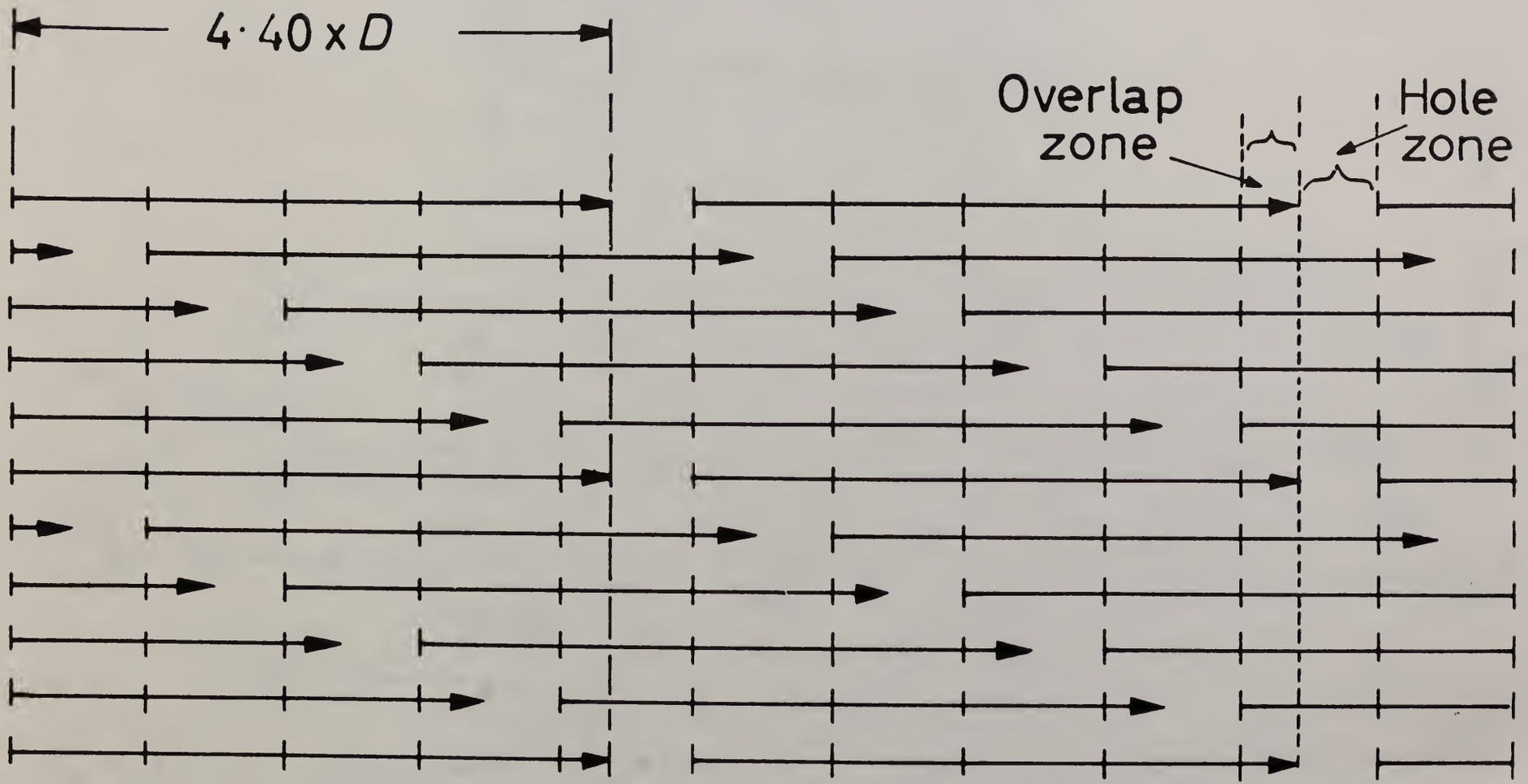


fig 5

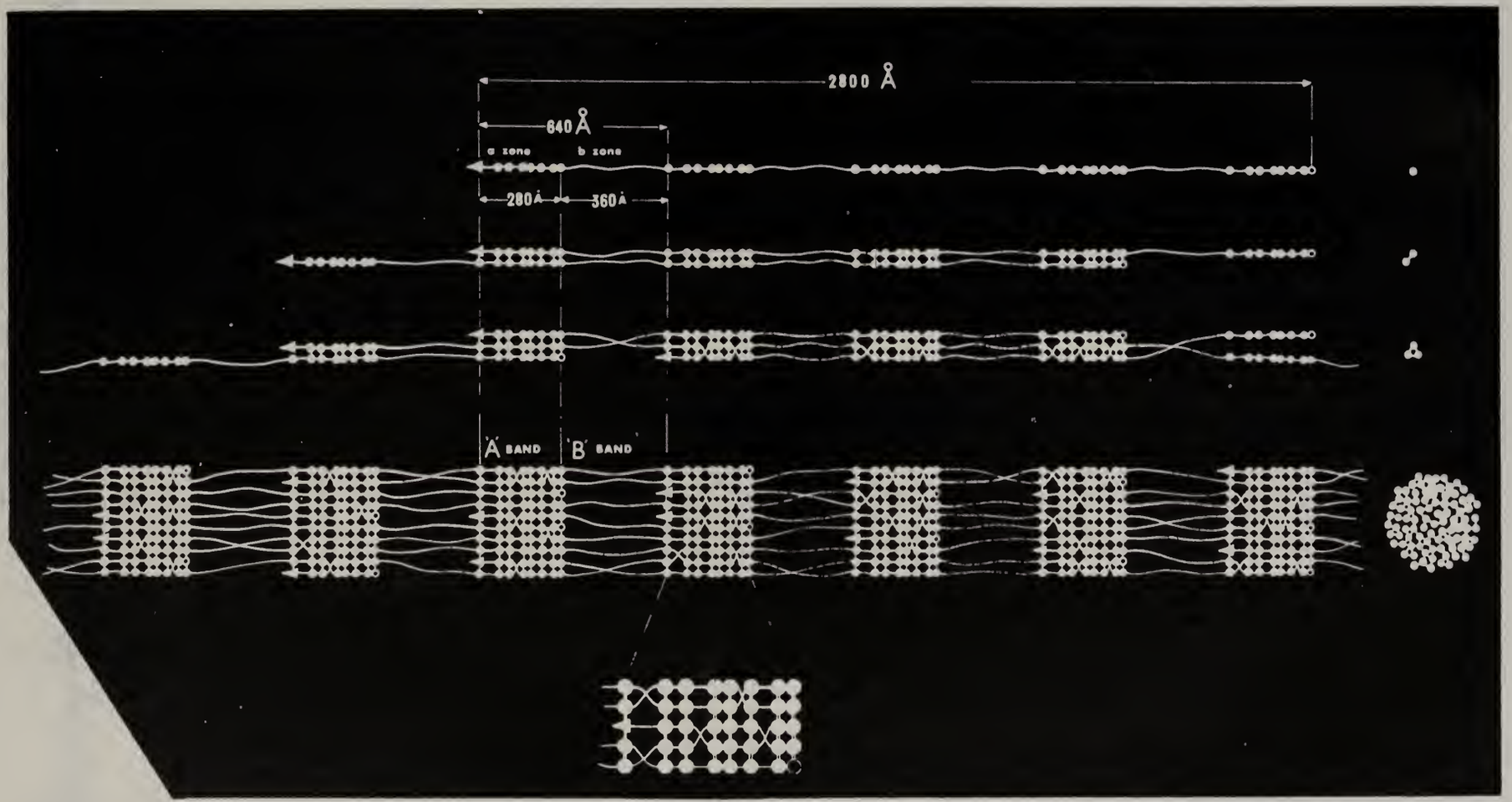


fig 6

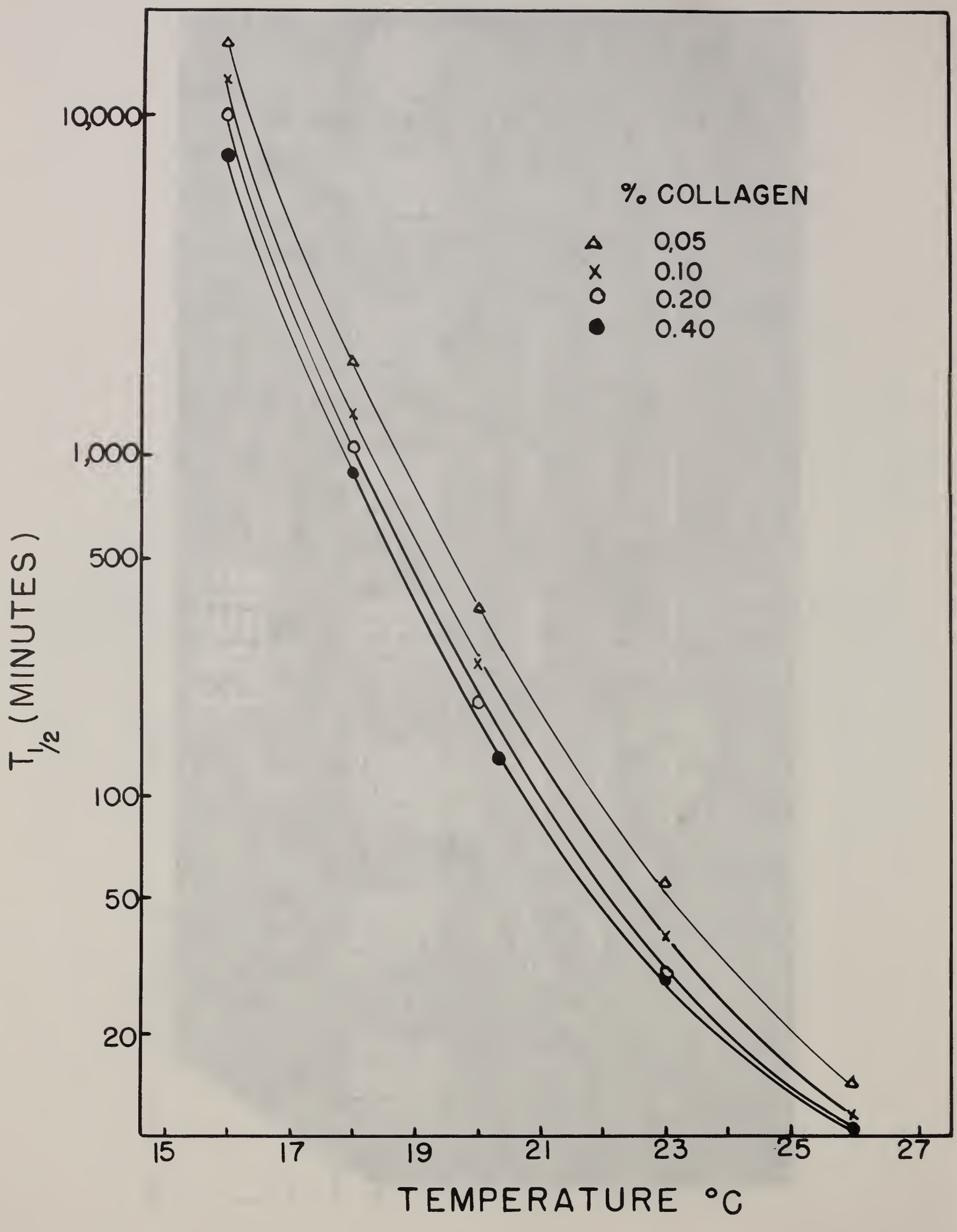

10,000
1,000
500
100
50
20
$T_{1/2}$ (MINUTES)
% COLLAGEN
0.05
0.10
0.20
0.40
15
17
19
21
23
25
27
TEMPERATURE °C

Fig 7

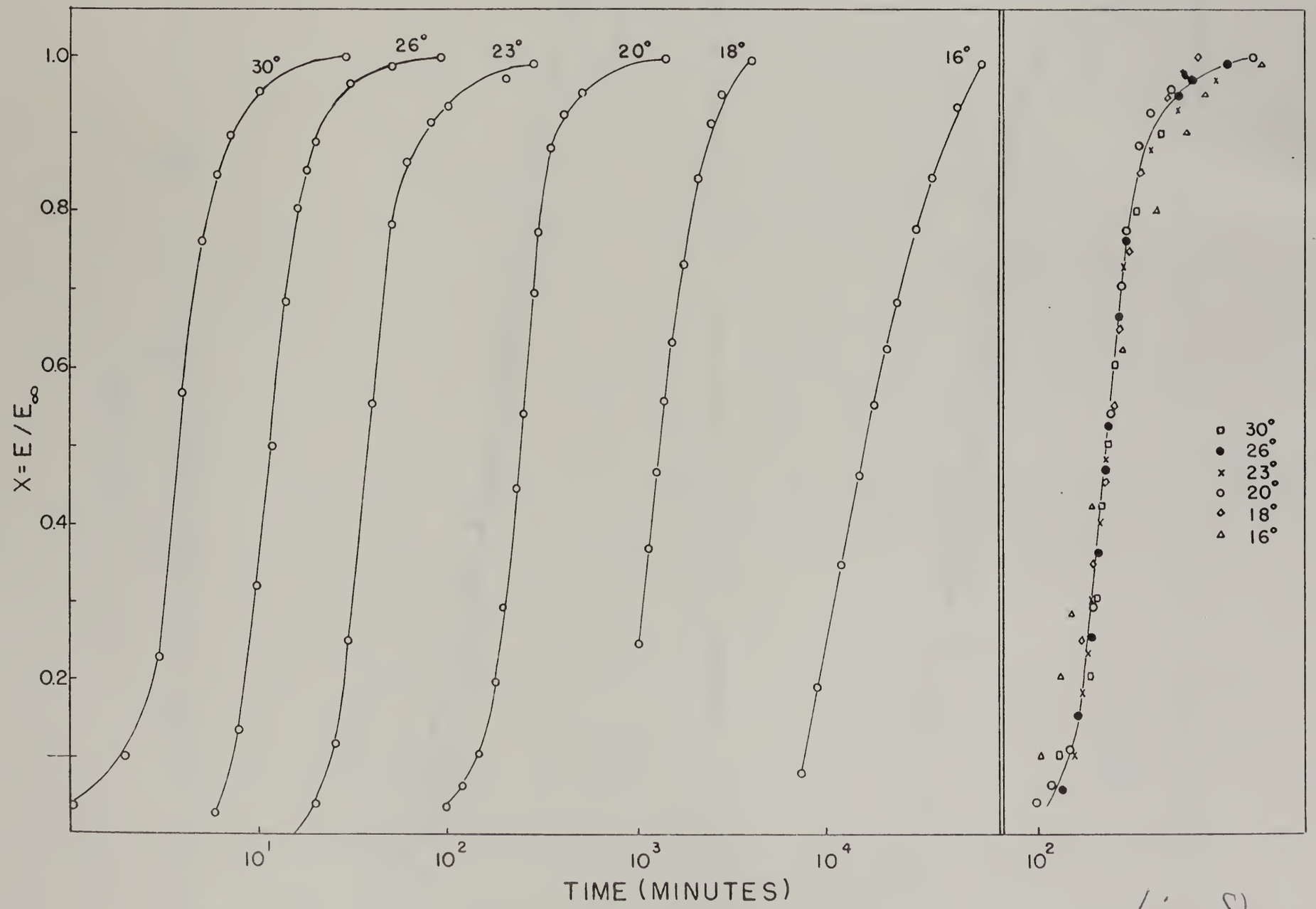

30°
26°
23°
20°
18°
16°
1.0
0.8
0.6
0.4
0.2
X = E / E∞
10¹
10²
10³
10⁴
TIME (MINUTES)
10²
30°
26°
23°
20°
18°
16°

fig 8

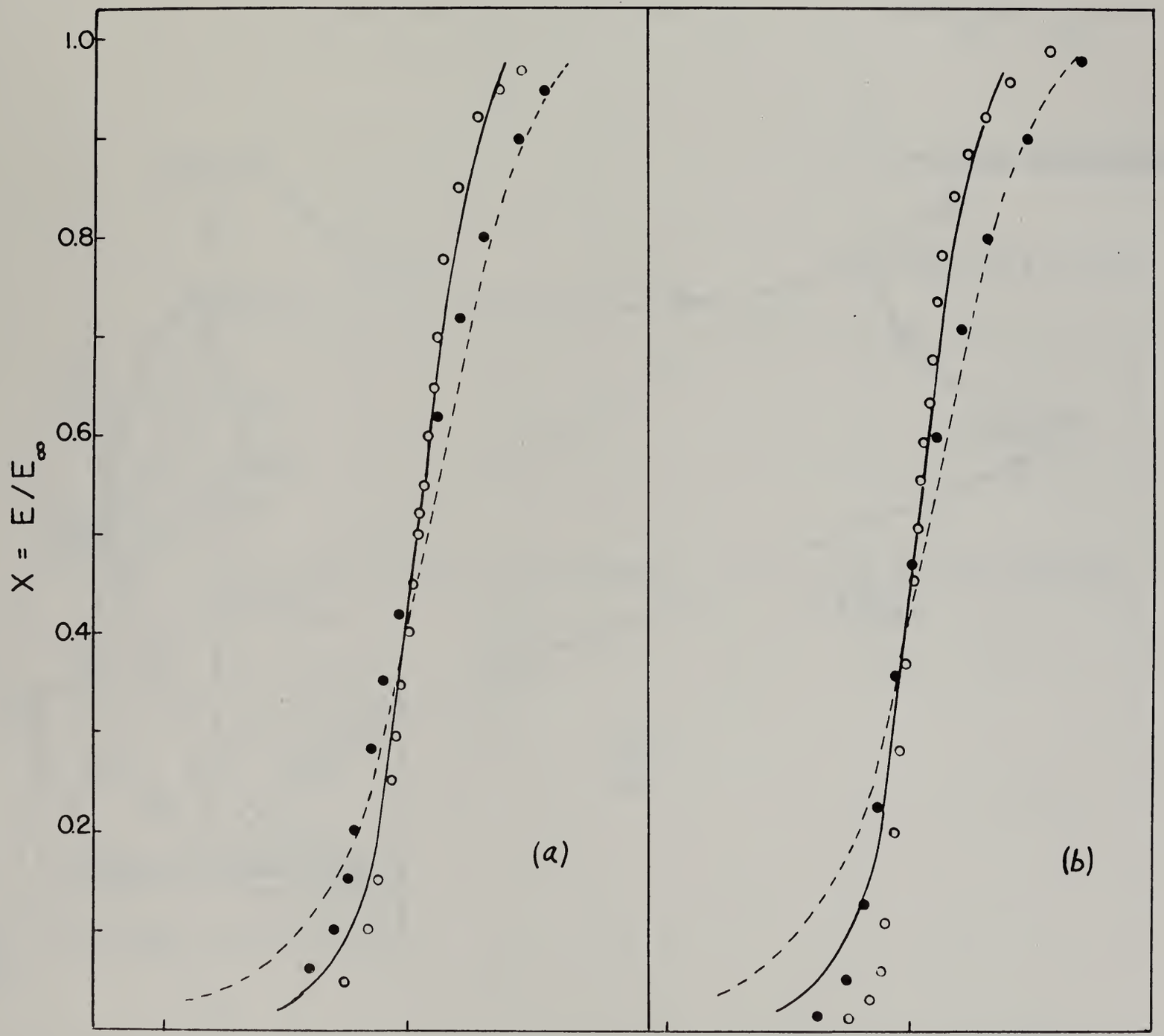


Fig 9

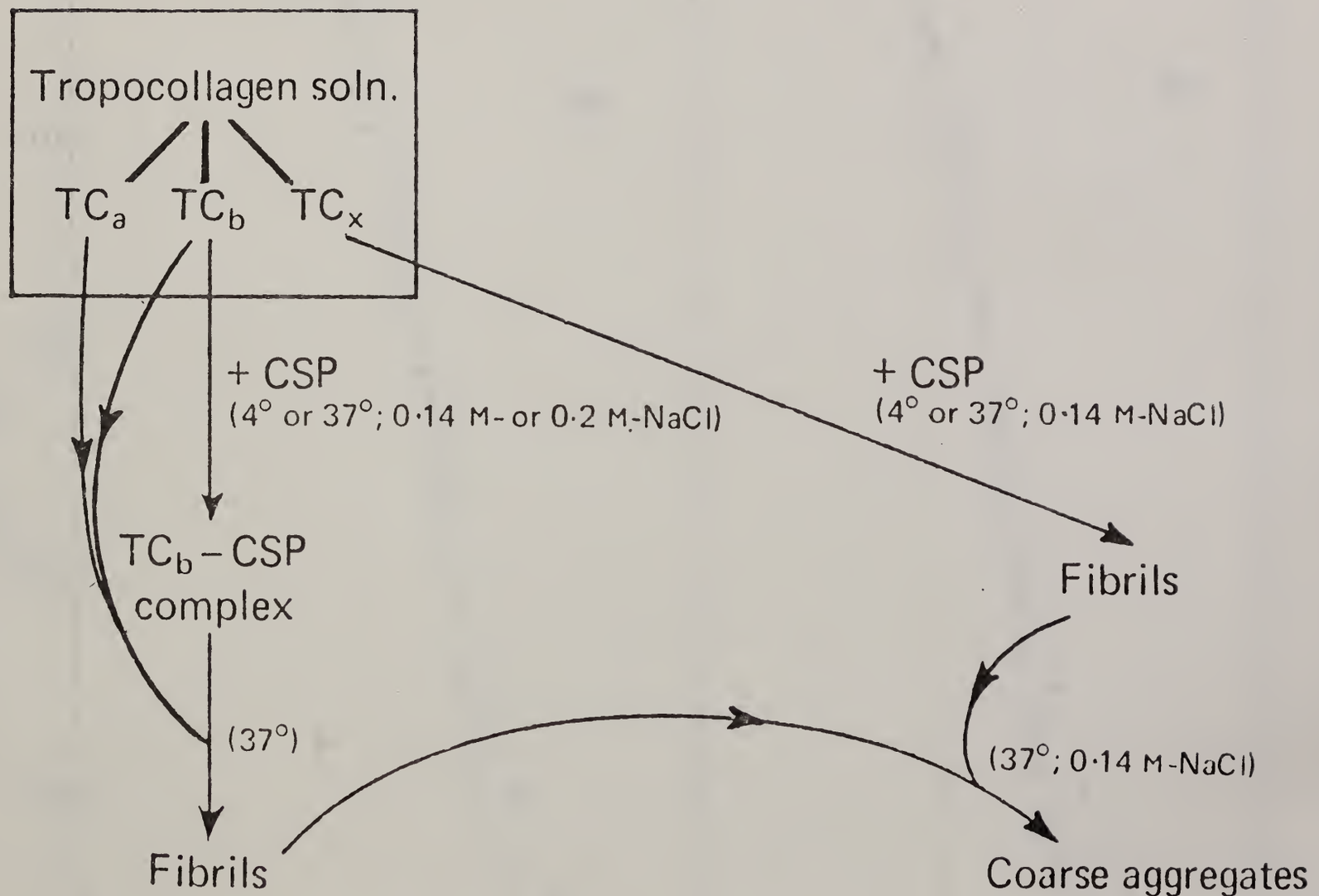

Tropocollagen soln.
TC_a
TC_b
TC_x
+ CSP
(4° or 37°; 0·14 M- or 0·2 M-NaCl)
+ CSP
(4° or 37°; 0·14 M-NaCl)
TC_b – CSP
complex
Fibrils
(37°)
(37°; 0·14 M-NaCl)
Fibrils
Coarse aggregates

fig 10

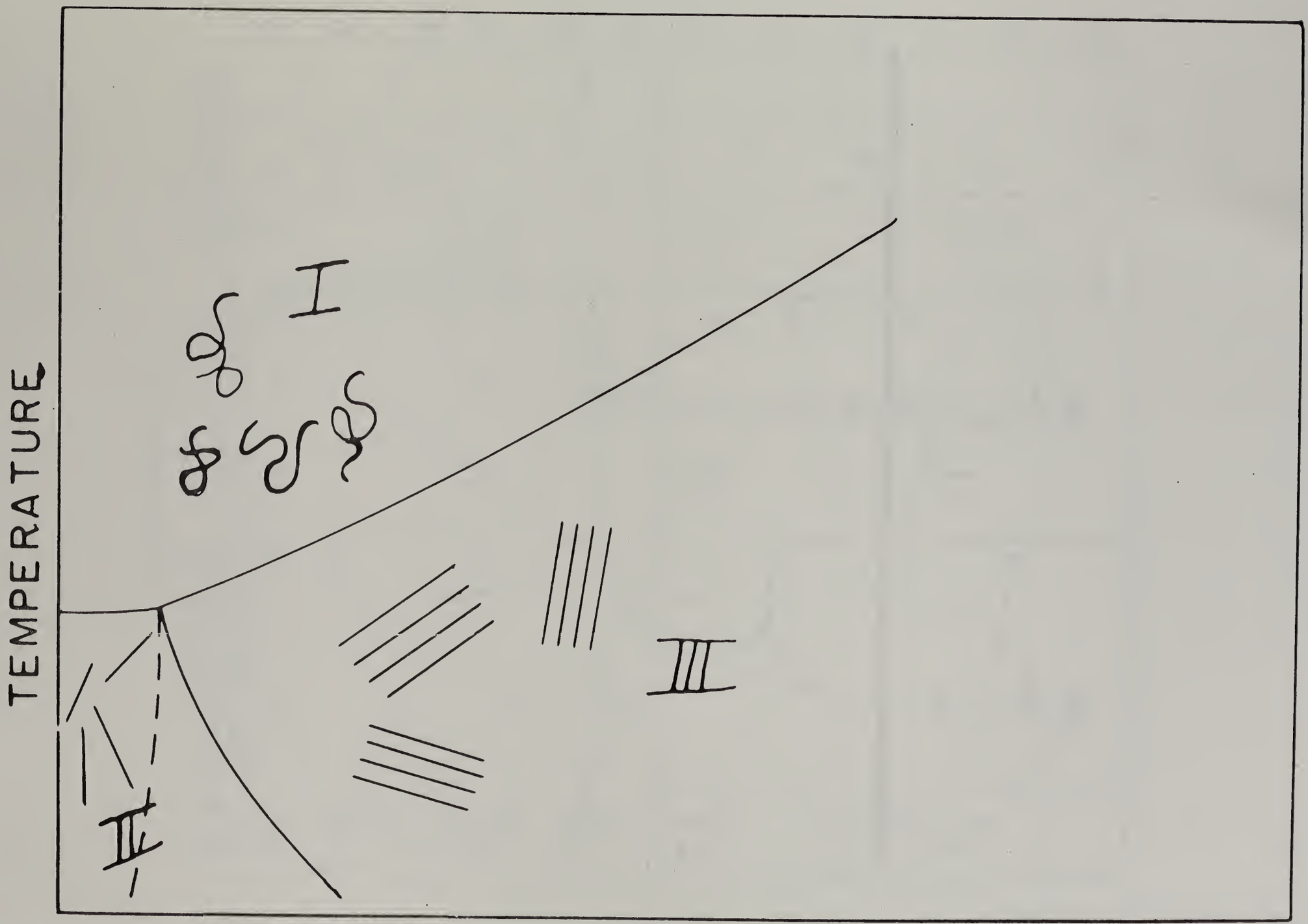


fig 11

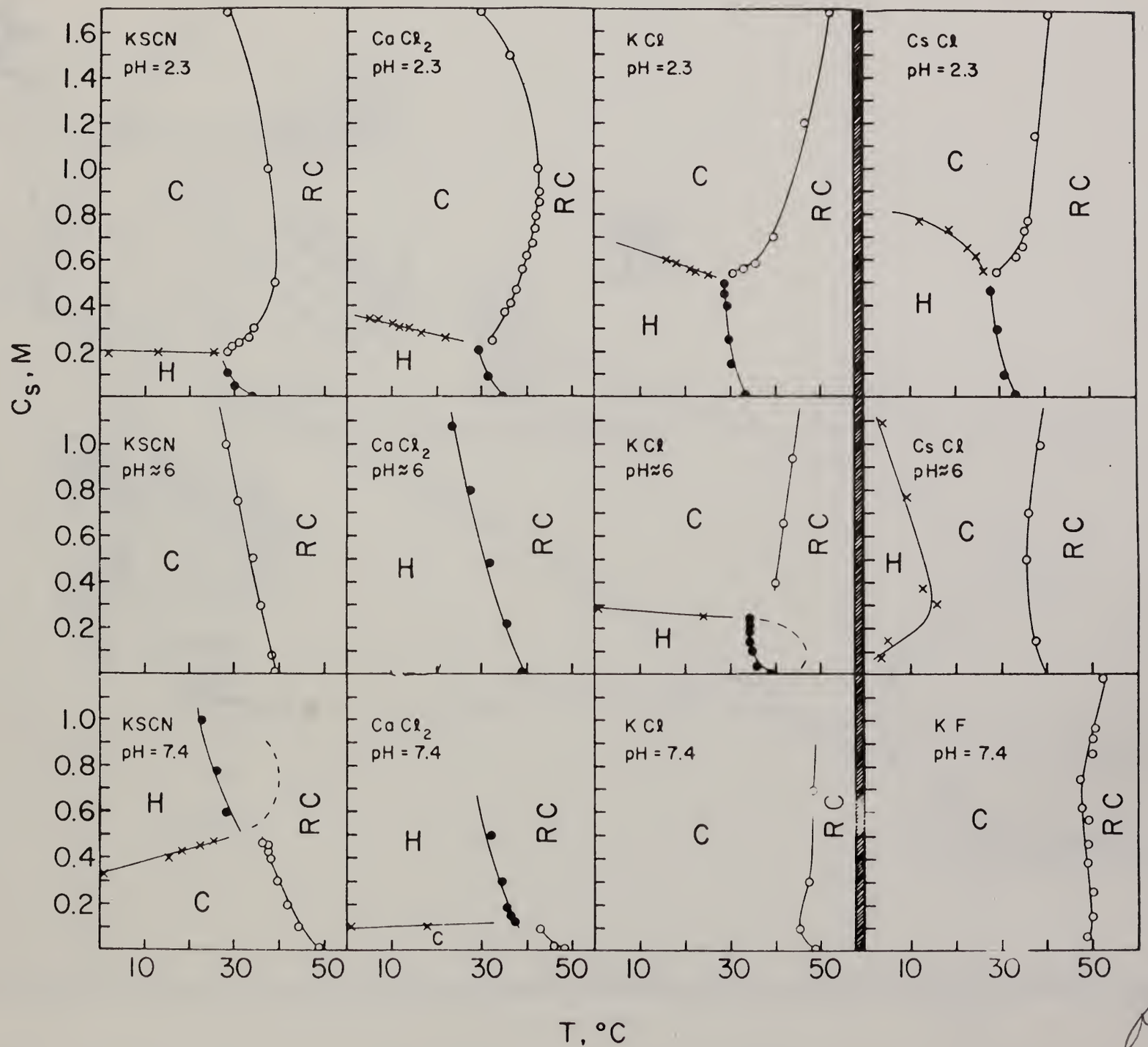

KSCN pH = 2.3
CaCl2 pH = 2.3
KCl pH = 2.3
CsCl pH = 2.3
KSCN pH ≈ 6
CaCl2 pH ≈ 6
KCl pH ≈ 6
CsCl pH ≈ 6
KSCN pH = 7.4
CaCl2 pH = 7.4
KCl pH = 7.4
KF pH = 7.4
C
H
RC
Cs, M
T, °C
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
10
30
50

fig 12

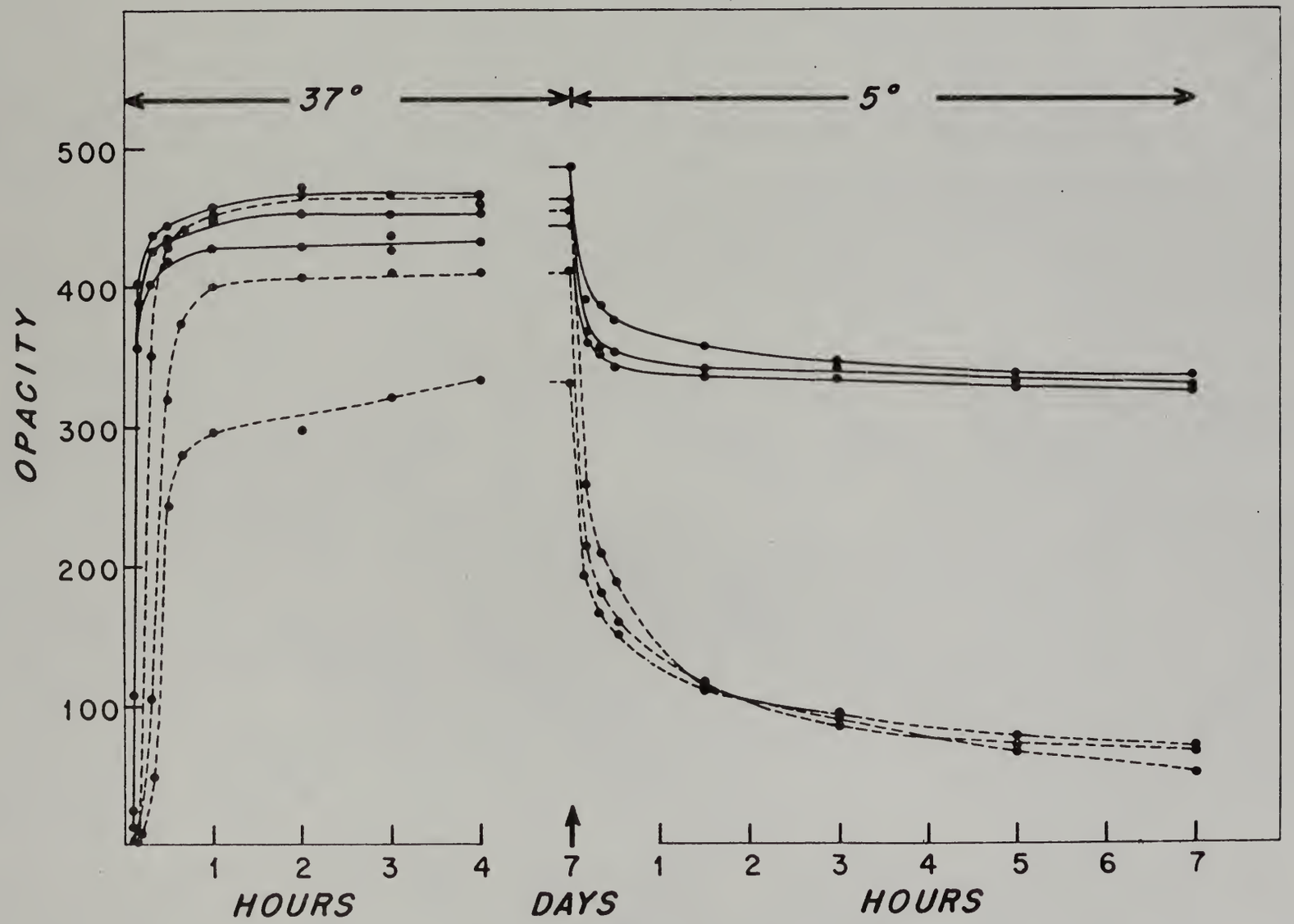

37°
5°
500
400
300
200
100
OPACITY
1
2
3
4
HOURS
7
DAYS
1
2
3
4
5
6
7
HOURS

fig 13