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The Formation of Fibrils from Collagen Solutions*

1. THE EFFECT OF EXPERIMENTAL CONDITIONS: KINETIC AND ELECTRON-MICROSCOPE STUDIES

BY G. C. WOOD† AND M. K. KEECH‡
Department of Medicine, University of Leeds

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The idea has grown over the last decade that study of the precipitation of collagen fibrils from collagen solutions *in vitro* can provide some insight into the mechanism of fibrillogenesis *in vivo*. The formation and growth of collagen fibrils in tissue cultures has been widely studied by electron microscopy (Porter & Vanamee, 1949; Porter, 1951; Jackson, S. F., 1954*a*, *b*, 1955, 1956; Wassermann, 1954). Although it still seems to be an open question whether the fibrils are initially formed within or outside the fibroblasts, these studies indicate that the fibrils grow by accretion of soluble collagen present in the ground substance.

Soluble collagens may be extracted from young collagenous tissues by cold neutral-salt solutions, slightly alkaline buffers, dilute organic acids or organic acid buffers. Typical collagen fibrils may be precipitated, from solutions obtained, by suitably altering the experimental conditions (see Jackson, 1958*a*, for a review). They may also be formed by adding to the collagen solutions low concentrations of certain mucopolysaccharides and these observations have led to suggestions that mucopolysaccharides, abundant in developing connective tissue, might play an important role in the organization of soluble collagen precursors into fibrillar form (Meyer, 1946, 1955–56; Gross, 1956). There is, however, no strong evidence that their presence is essential for the formation of collagen fibres *in vivo* (Jackson, 1958*b*). Gross, Highberger & Schmitt (1955) and Jackson & Fessler (1955) have shown that collagen may be precipitated in the 640 Å striated form typical of native collagen, in the absence of added mucopolysaccharide, suggesting that these substances may not be important in this stage of the formation of collagen fibres. As indicated by Gross (1956), they may, however, play a part in the further organization of the fibrils.

Like the studies of fibril growth *in vivo*, most of the work on precipitation of collagen *in vitro* has been largely concerned with the appearance of the material in the electron microscope. Much of it has been qualitative and, except for the early work of Vanamee & Porter (1951), no systematic study has been made of the factors affecting precipitation, although Randall, Booth, Burge, Jackson & Kelly (1955) have enumerated the various factors to be considered.

Little attention has been paid to the kinetics of collagen precipitation. While the present work was in progress, however, Gross (1956), Gross & Kirk (1958) and Bensusan & Hoyt (1958) reported observations on factors affecting the rate of precipitation of collagen fibrils from neutral-salt solutions. The course of the precipitation was followed by measuring the increase in opacity of the system. In the present study a similar technique has been applied to a systematic study of the effect of experimental conditions (pH, ionic strength, temperature and collagen concentration) on the rate of fibril formation. Parallel to the rate studies the morphology of the collagen precipitates has been investigated by electron microscopy, the aims being to ascertain that the precipitates consisted of fibrils bearing 640 Å cross-striations typical of native collagen and to see how the size of the ultimate fibrils varied with the rate of precipitation under different conditions. The results provide a basis for formulating a kinetic mechanism which is treated in greater detail in the next paper (Wood, 1960*a*).

It seems likely that, as suggested by Gross (1958), solutions prepared by the different extractive procedures differ mainly in the state of aggregation of the dissolved collagen particles and that in each case the process of precipitation is essentially the same. In the present study acid-soluble collagen has been used, prepared in a form substantially free of mucopolysaccharide. The further problem of the effect of added mucopolysaccharides on fibril formation is considered by Wood (1960*b*).

A preliminary account of some of this work has been published (Wood, 1958).

* A paper by one of us (G.C.W.) based on work described in this and the following two papers gained second place in the Ciba Foundation's Awards, 1959, for Research Relevant to the Problems of Ageing.

† Nuffield Gerontological Research Unit.

‡ Empire Rheumatism Council Research Fellow.

EXPERIMENTAL

Materials

Purified calf dermis. Fresh calf skin was defatted mechanically and cut into strips. Hair, epidermis and remaining subcutaneous tissue were removed by dissection and the strips chopped into small pieces. These were passed through a power mincer, solid CO_2 being used as refrigerant, and homogenized with 3 vol. of water and freeze-dried. Most of the contaminating proteins, mucopolysaccharide and some soluble collagen were removed by neutral-salt extraction as follows: 50 g. of dermis was stirred gently with 650 ml. of 0·2 M-NaCl, pH 7·0, for 16 hr. Undissolved material was separated by centrifuging and extracted again for 16 hr. with 500 ml. of 0·2 M-NaCl. The residue was treated twice in the same way with 1·0 M-NaCl, pH 7·0, and once with saturated NaCl. The final residue was washed with water until the washings were free of salt, suspended in water and freeze-dried. All the operations were carried out at 0–4° and the resulting purified dermis was stored at this temperature. For all the experiments described in this paper a single preparation of purified dermis (dermis X) was used.

Collagen solutions. Purified dermis (1 g.) was mixed with 60 ml. of 0·5 M-acetic acid and allowed to stand for 16 hr. with occasional gentle stirring. The mixture was filtered through a coarse sintered-glass filter and the clear filtrate dialysed for a total period of 48 hr. against four changes of 2 l. of 0·1 M-NaCl containing 0·005 M-sodium acetate buffer, pH 4·2. The resulting viscous solution was then centrifuged for 45 min. at 20 000 *g*. All the operations were carried out at 0–4° and the collagen solution was stored at this temperature after its concentration had been adjusted to 0·1 % by dilution with cold 0·1 M-NaCl. Analytical data for a typical solution are given in Table 1. Assuming that the hydroxyproline content of collagen is 8·2 g. of amino acid N/100 g. of N (Bowes, Elliott & Moss, 1955), the hydroxyproline content of the solution indicates that about 93 % of the dissolved protein is collagen.

Examination of a dried collagen solution prepared from another similar sample of purified dermis showed (Plate 1, photograph 1) that it consisted of non-striated filaments of varying size. No striated collagen fibrils were seen.

Analytical methods

Hydroxyproline was determined by the method of Neuman & Logan (1950) and nitrogen by the micro-Kjeldahl method. Hexosamine was estimated as glucosamine by the method of Boas (1953), hexose by the anthrone method of Scott & Melvin (1953) and phosphorus by the method of King (1932). Routine protein estimations were done by the method of Lowry, Rosebrough, Farr & Randall (1951). Viscosities were measured at 25° in Ostwald–Fenske viscometers with flow times for water of about 6 min.

Table 1. *Analytical data for soluble collagen prepared from purified calf dermis*

Component	g./100 g. of total N
Hydroxyproline N	7·6
Hexosamine (as glucosamine)	1·7
Hexose (as glucose)	1·3
Phosphorus	0·13

Methods of following the course of precipitation

Turbidity method. In a typical experiment 1·5 ml. of collagen solution was allowed to stand in a spectrophotometer cell (1 cm. light path) at 25° for 1·5 hr. At zero time precipitation was initiated by adding 1·7 ml. of buffer solution (pH range 6–8) previously equilibrated at 25° and mixing the cell contents thoroughly with a polythene stirrer similar to that described by Dixon (1954). The stirrer was withdrawn and E at 400 mμ measured at intervals throughout formation of the gel-like precipitate. A Unicam SP. 500 spectrophotometer fitted with a constant-temperature cell holder was used. It was modified by placing a pin hole (approx. 0·5 mm. diameter) in front of the dark current shutter in order to minimize the amount of forward-scattered light reaching the photocell. One result of this was to increase the apparent extinction of a gel by 15–20 %.

NaOH–KH_2PO_4 buffers were used, the total phosphate content being maintained constant so that its value in the reaction mixture was 0·04 M. When the effect of pH was studied the ionic strength was maintained by adjusting the NaCl content of the buffer. To study the effect of ionic strength (I) the NaCl content of the added buffer was varied.

In all experiments the collagen solution was equilibrated at a concentration of 0·1 % at 25°, well below the thermal-transition temperature reported by Doty & Nishihara (1958). For experiments at temperatures other than 25° the buffer was equilibrated at the working temperature, added to the collagen solution at 25° and the mixture brought as quickly as possible to working temperature. For experiments at different collagen concentrations different volumes of 0·1 % collagen solution were equilibrated and the volume and composition of the added buffer adjusted so that the final volume and salt content were the same.

Fig. 1 shows progress curves for two successive experiments which give an indication of the reproducibility. Other work showed that the curves changed gradually with the age of the collagen solution. For this reason only solutions less than 10 days old were used and progress curves are strictly comparable only when the experiments were done on the same day. Some variation from one preparation of collagen solution to another was observed.

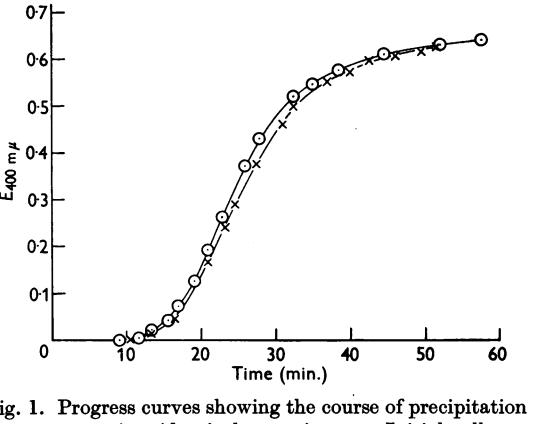


Fig. 1. Progress curves showing the course of precipitation in two successive, identical, experiments. Initial collagen concn., 0·05 %; temp., 25°; I 0·23; pH 7·1.

Calibration of the turbidity method by a chemical procedure. Since the relationship between extinction and amount of precipitate cannot be predicted theoretically it was necessary to calibrate the method by actually measuring the amount of precipitate. Simultaneously with a turbidity experiment several precipitations were allowed to proceed, under the same conditions, in 10 ml. centrifuge tubes. After different known reaction times 0·2 ml. of 0·5 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) hydrochloride in 0·2 M-NaOH–KH_2PO_4 buffer, pH 7, was added. The collagen gel was compressed to the bottom of the tube, centrifuged and the protein content of the supernatant estimated, enabling the extent of precipitation to be calculated and compared with the extinction of the gel at the corresponding time. Addition of tris to the reaction mixture prevented any significant further precipitation during the time required for the estimation and dissolved less than 3% of the collagen already precipitated.

Fig. 2 shows the relationship between the extinction of a collagen gel at different times during its formation and the corresponding extent of precipitation, p, given by $p = (C_0 - C)/(C_0 - C_s)$, where C is the collagen concentration. C_0 and C_s are the initial and final collagen concentrations respectively. Thus $p = E/E_\infty$ to a close approximation, where E is extinction and E_∞ the extinction at infinite time. Similar straight lines were obtained in several experiments under different conditions. Extinction is therefore a reliable measure of the extent of precipitation and, in view of its convenience and precision, the turbidity method has been used for the results described below.

Electron-microscope methods

Preparation and examination of collagen precipitates. The collagen solutions and buffers were the same as those used for the rate studies and as far as possible the procedure for preparing the precipitates was also the same.

Glass slides were equilibrated in Petri dishes maintained at working temperature (25°, 37° or bench temperature—approx. 21°). After the collagen and buffer solutions had been equilibrated at the required temperatures and mixed as described in the rate studies, approximately 0·5 ml. of the mixture was immediately transferred to duplicate slides, spread to make pools 1–2 mm. deep and immediately replaced in the Petri dishes. Extra buffer in tinfoil cups was put in each dish to minimize evaporation of water from the reaction mixtures.

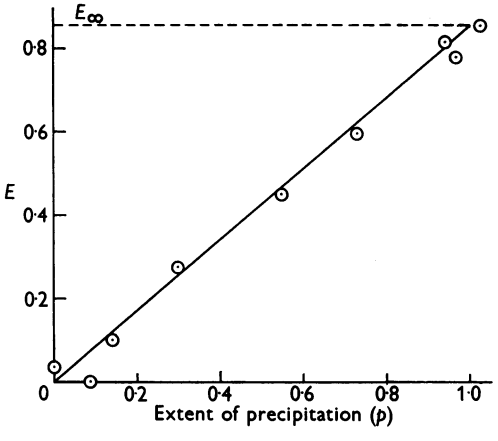


Fig. 2. Relationship between E of a collagen gel at different times during its formation and the extent of precipitation (p) determined chemically. E_∞, final extinction. Initial collagen concn., 0·05%; temp., 25°; I 0·23; pH 7·1.

After 24 hr. the slides were dried in 10–15 min. in a stream of air at room temperature. They were gently washed with water to remove buffer salts, dried again, shadowed with gold–palladium at an angle of 5° and covered with a thin film of collodion. After scoring the film into squares, dermal components were removed by trypsin digestion overnight at room temperature. The loosened squares were then peeled from the slide, washed in a dish of water and picked up on electron-microscope grids. The final preparations thus consisted of metal-shadowed replicas of the surfaces of the undisturbed, washed, dried precipitates. These were examined in a Siemens electron microscope, type UM 60C, large areas of several grids from each preparation being scrutinized.

No antibacterial agents were used but, by employing only freshly-made collagen and buffer solutions, bacterial contamination was avoided.

Measurement of fibril width. In conformity with present-day terminology the term 'fibril' denotes a fibrous structure which is apparently undivided at the magnification used.

Fibril width was measured on the electron-microscope plates with a scaled Leitz eyepiece (magnification × 8). Only single fibrils were measured, aggregates of fibrils being disregarded. It is assumed that the former provide a representative sample of all the fibrils.

RESULTS

Effect of thermal and mechanical agitation of the collagen solutions

Fig. 3 shows how precipitation curves for one collagen solution varied with the time of equilibration of the solution at 25° and indicates that 1·5 hr. is required to attain true equilibrium. This is much greater than the time required to attain temperature equilibrium and must be due to some change in the state of the dissolved collagen. This change is accompanied by a change of viscosity, as indicated in Fig. 3. Similar effects were caused by stirring the extract at 0°, when they were most pronounced in dilute solutions and were partly reversed by allowing the solution to stand for 16 hr. at 0°. These phenomena serve to emphasize the necessity of adhering to the strict routine already described in performing the precipitation experiments, minimizing mechanical agitation of the collagen and always using an equilibration time of 1·5 hr.

Effect of varying collagen concentration, pH, ionic strength and temperature

Rate of precipitation. In Figs. 4 and 5 the four sets of precipitation curves show the effect of each of the variables in turn.

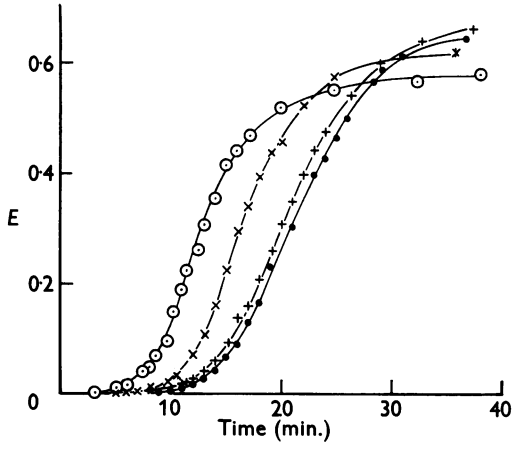


Fig. 3. Effect of varying time of equilibration (t_e) on subsequent precipitation. t_e: ⊙, 0·5 hr.; ×, 1·0 hr.; ●, 1·5 hr.; +, 3·25 hr. Relative viscosities ($\eta_{rel.}$) of corresponding equilibrated collagen solutions: 2·98, 2·84, 2·75 and 2·73 respectively. During precipitation: initial collagen concn., 0·05%; temp., 25°; I 0·23; pH 7·1.

All the precipitation curves have the same shape, consisting of a lag period, during which no precipitation was observed, followed by a sigmoid portion or growth period. Although it cannot be determined precisely, the extent of the lag period is approximately given by $t_{0\cdot005}$, the time taken for p to rise to the value 0·005. A measure of the rate of growth is given by the half-growth time ($t_{0\cdot5}$), i.e. time taken for p to rise from 0·005 to 0·5. These two quantities are given in Tables 2–4 for the above data, together with the results of some experiments not given in graphical form. E_∞/C_0 is also shown in the tables. Since $C_s \simeq 0$ in all experiments (i.e. the collagen was completely precipitated) the results are comparable within each set.

In all cases a change of experimental conditions which shortened the lag period also accelerated subsequent precipitation and vice versa. Precipitation was accelerated by increasing the collagen concentration and by increasing the temperature. It was retarded by increasing the ionic strength at pH 7 and 8; at pH 6, change of ionic strength from 0·13 to 0·23 retarded precipitation but further increase to 0·31 accelerated it. When pH was varied at ionic strength 0·23 the rate of precipitation showed a minimum at pH 7·0–7·5.

Acceleration of precipitation by changing the ionic strength or temperature was accompanied by

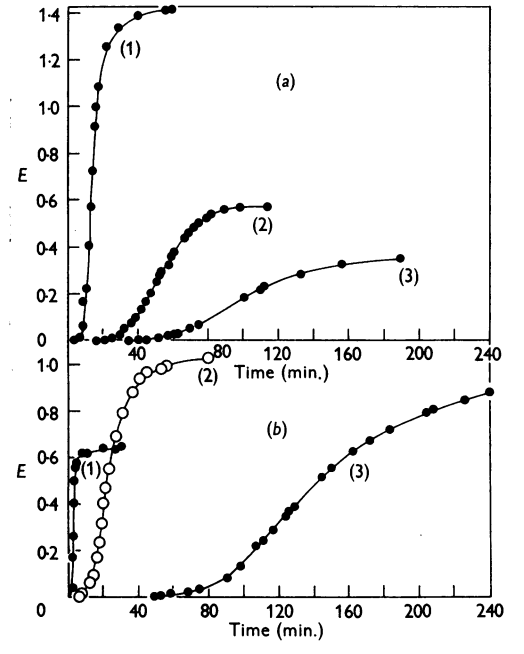


Fig. 4. Effect of varying experimental conditions on precipitation. (*a*) Different initial collagen concentrations (C_0): (1) 0·084; (2) 0·028; (3) 0·017%. (*b*) Different temperatures: (1) 37·0°; (2) 25·0°; (3) 20·2°. Except for the single variables in each set, C_0 was 0·05%; temp., 25°; I 0·23; pH 7·1. The two sets of experiments were done on different collagen solutions.

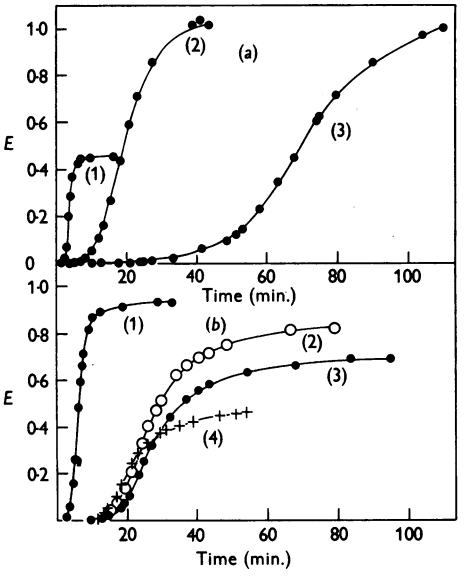


Fig. 5. Effect of varying experimental conditions on precipitation. (*a*) Different ionic strengths: (1) I 0·13; (2) I 0·23; (3) I 0·31. (*b*) Different pH values: (1) pH 6·0; (2) pH 7·1; (3) pH 7·5; (4) pH 8·0. Except for the single variables in each set, C_0 was 0·05%; temp., 25°; pH 7·1; I 0·23. The two sets of experiments were done on different collagen solutions.

Table 2. *Rate of precipitation of collagen at different initial concentrations (C_0) of collagen*

Each set of data was obtained with a different preparation of collagen solution. Experimental conditions: pH 7·1; I 0·23; 25°. For definition of $t_{0·005}$, $t_{0·5}$ and E_∞ see text.

C_0 (%)	Lag period, $t_{0·005}$ (min.)	Half-growth time, $t_{0·5}$ (min.)	E_∞/C_0
0·084*	3	12	16·8
0·061	8	14	21·5
0·050	8	12	19·0
0·028*	20	35	15·8
0·017*	30	73	21·5
0·050	5	12	21·0
0·034	13	39	21·7
0·017	30	87	21·2

* Data correspond to curves in Fig. 4*a*.

Table 3. *Rate of precipitation of collagen at different values of* pH *and ionic strength*

Each set of data was obtained with a different preparation of collagen solution. C_0 0·05%; 25°. For definitions of $t_{0·005}$, $t_{0·5}$ and E_∞ see text.

pH	I	Lag period, $t_{0·005}$ (min.)	Half-growth time, $t_{0·5}$ (min.)	E_∞/C_0
6·0*	0·23	2	5	18·8
7·1*	0·23	8	20	16·6
7·5*	0·23	12	18	14·0
8·0*	0·23	9	13	9·2
6·0	0·23	2·5	2·7	18·0
7·1	0·23	6	10	18·0
8·0	0·23	5	8	9·2
7·0†	0·13	2	2	9·2
7·0†	0·23	5	15	21·6
7·0†	0·31	24	48	23·0
7·0	0·13	2	1·5	8·0
7·0	0·23	6	10	18·0
7·0	0·31	13	22	18·8
6·0	0·13	1	2·5	11·8
6·0	0·23	2	4·5	18·8
6·0	0·31	1	3	19·8
8·0	0·13	3	5	6·8
8·0	0·23	6	7	9·4
8·0	0·31	20	50	16·4

* Data correspond to curves in Fig. 5*b*.
† Data correspond to curves in Fig. 5*a*.

Table 4. *Rate of precipitation of collagen at different temperatures*

Experimental conditions: pH 7·0; I 0·23. For definitions of $t_{0·005}$, $t_{0·5}$, E_∞ and C_0 see text. Data correspond to curves in Fig. 4*b*.

Temp.	Lag period, $t_{0·005}$ (min.)	Half-growth period, $t_{0·5}$ (min.)	E_∞/C_0
20°	45	93	18·0
25	9	19	21·0
30	3	9	23·6
37	1	2	13·0

a decrease of E_∞/C_0. On the other hand, E_∞/C_0 fell continuously as pH was increased from 6 to 8 and does not seem to be correlated with the rate of precipitation in a simple manner. E_∞/C_0 was essentially independent of collagen concentration. Since the collagen was always completely precipitated, the difference of E_∞/C_0 observed in each set of experiments must be due to differences in the structure of the precipitates. The nature of these differences will become clear when the electron-microscope results are described.

Tables 2–4 also show the variation observed in precipitation behaviour from one preparation of collagen solution to another. In spite of this, the manner in which rate of precipitation varied with conditions was reproducible.

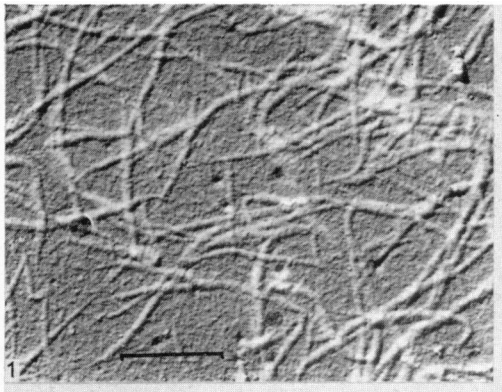
Electron-microscope results. Examination of the replicas under low power (× 300) showed that the precipitates consisted of fibrous networks whose coarseness varied with experimental conditions. These networks were seen under high power (× 10 000) to consist almost entirely of long fibrils bearing 640 Å striations (Plates 1 and 2, photographs 2–9) together with variable quantities of background filaments. The width of the fibrils varied with experimental conditions.

The fibrils showed varying degrees of lateral aggregation. Bundles of fibrils (e.g. Plate 1, photograph 3) occurred in most of the precipitates. Their component fibrils, usually 1000–1400 Å wide, were separate and the bundles, which frequently looked cylindrical, sometimes appeared to be twisted. Fibrils were sometimes observed side by side in one plane with their cross-striations in register. When wide fibrils were aggregated in this way, with

EXPLANATION OF PLATE 1

Plates 1 and 2. The electron micrographs are (except no. 1) of replicas of precipitates shadowed with gold–palladium. The scale on each photograph represents 1 μ. The striated structures exhibit 640 Å periodicity unless otherwise stated. Curves showing the rate of formation of the precipitates illustrated in photographs 2–4 and 7 and 8 are given in Figs. 4*c* and 9 respectively.

(1) The fibrous felt-work observed when a drop of the collagen solution was air-dried on the grid, washed and shadowed with chromium. (2–4) Precipitates prepared at different ionic strengths at pH 7·1, 25°. For distribution of fibril width see Fig. 6. (2) Dense deposit of fibrils with very little bundle formation, obtained at I 0·13. (3) Marked lateral aggregation of fibrils to form bundles at I 0·23. Few background filaments were seen. (4) Fibril bundles and acute fibril bends (4*a*) and tapered fibril ends (4*b*) observed at I 0·31. Some background filaments were seen. The bright borders to some of the fibrils are artifacts. (5) Precipitate prepared at I 0·23, 25°. For distribution of fibril width see Fig. 7. Dense deposit of fibrils with very little bundle formation, observed at pH 8·0.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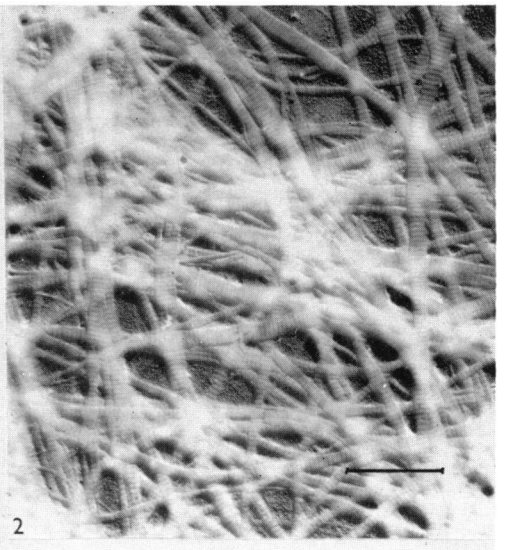
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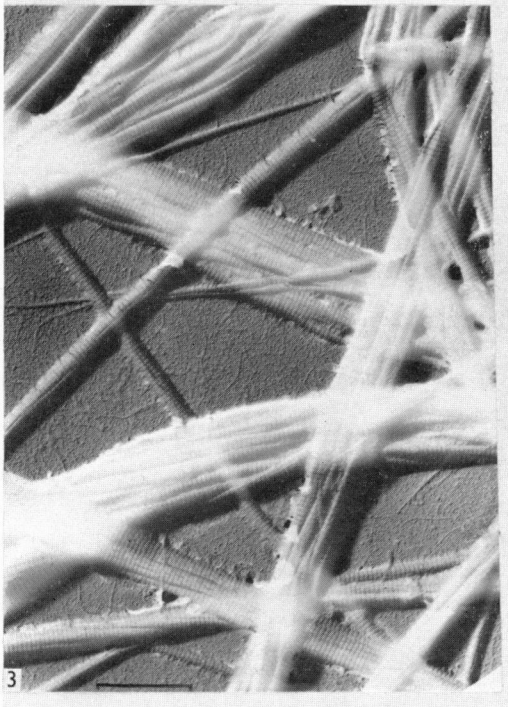
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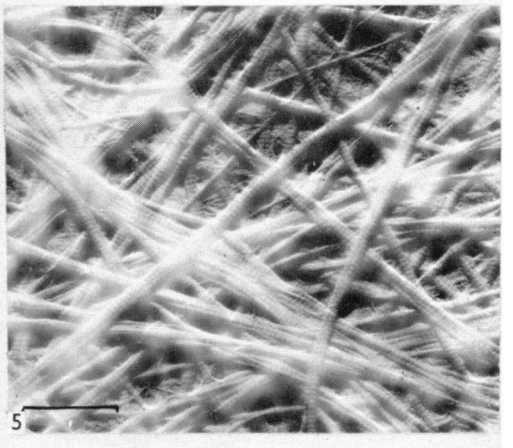
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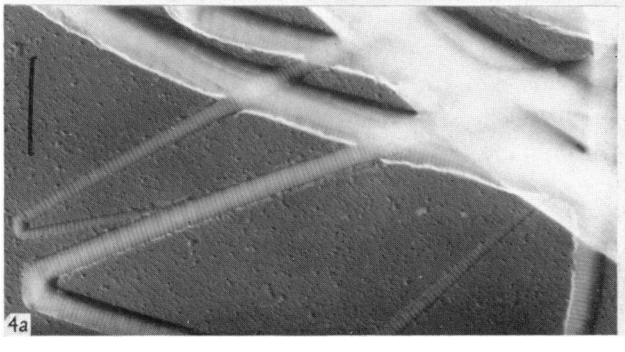
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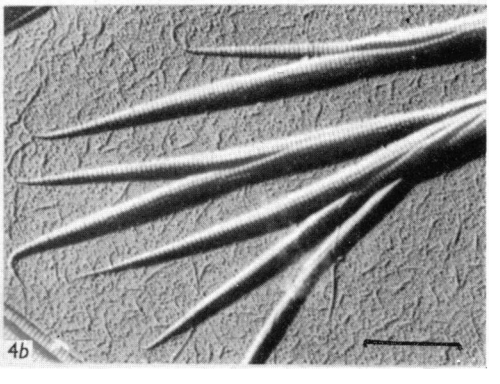
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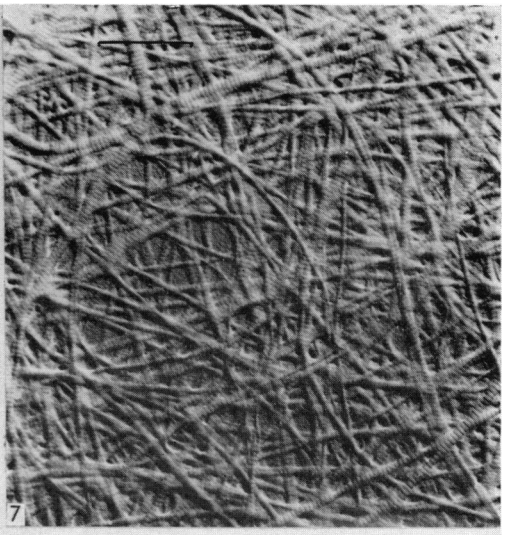
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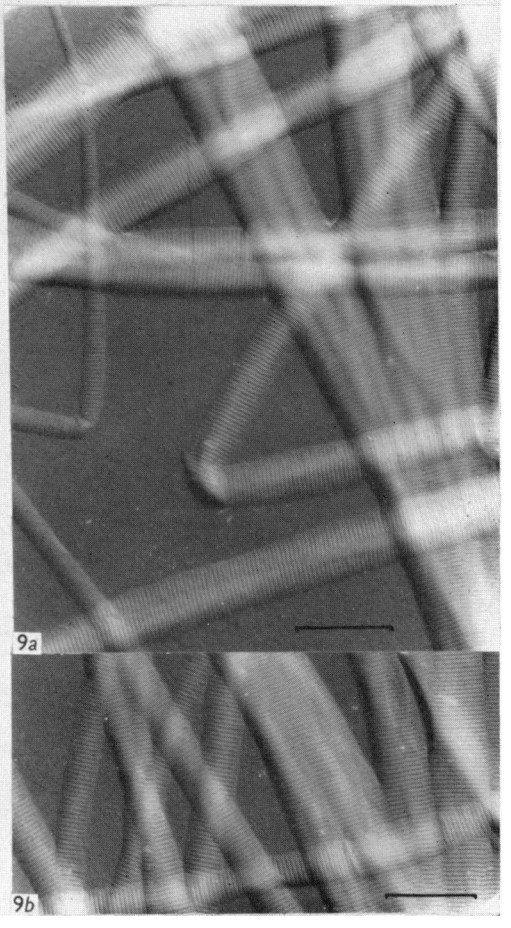
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9b

partial loss of identity, they gave the appearance of sheets (Plate 2, photograph 6) or compound fibres (Plate 2, photographs 9*a*, *b*). The above classification of lateral aggregation is arbitrary and there was in fact no sharp distinction between the various forms.

Table 5 summarizes the results of four sets of experiments designed to examine the effect of varying, in turn, each of the variables: ionic strength (*I*), pH and temperature. For each set a different preparation of collagen solution was used. The fibril-width data are taken from the histograms in Figs. 6–8 and Plates 1 and 2, photographs 2–9, show a representative set of electron-microscope photographs.

When ionic strength was varied at pH 7·1, 25°, the precipitate at *I* 0·13 appeared as a dense mat of discrete fibrils mainly 600–1200 Å wide (Plate 1, photograph 2). As *I* was increased to 0·23 and 0·31 the most frequent fibril width and the range of fibril width both increased (Fig. 6). Bundles were observed at *I* 0·23 (Plate 1, photograph 3) and at *I* 0·31, and at the latter ionic strength tapered fibril ends and acute fibril bends were seen (Plate 1, photographs 4*a*, *b*).

When pH was varied at constant *I* (0·23), 25°,

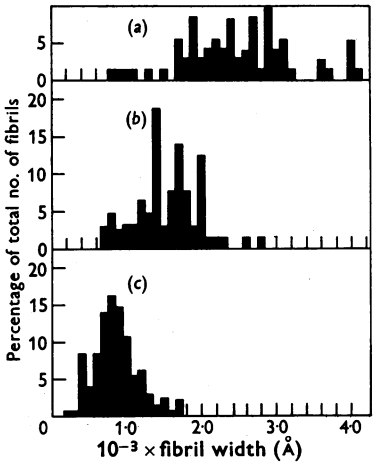

Fig. 6. Distribution of fibril width in precipitates prepared at different ionic strengths: (*a*) *I* 0·31; (*b*) *I* 0·23; (*c*) *I* 0·13. C_0, 0·05%; temp., 25°; pH 7·1. The number of fibrils measured was 72, 64 and 129 respectively. The data were obtained from the series of electron micrographs of which Plate 1 (2–4) gives examples.

EXPLANATION OF PLATE 2

(6) Precipitate prepared at *I* 0·23, 25°. For distribution of fibril width see Fig. 7. Sheet formation, which, together with compound fibres and bundles, was observed at pH 7·1. (7–9) Precipitates prepared at different temperatures at pH 7·1, *I* 0·23. For distributions of fibril width see Fig. 8. (7) Dense deposit of narrow fibrils obtained at 37°. No bundles were seen. Some of the narrower fibrils exhibited 220 Å striations. (8) Precipitate containing fibrils both singly and in bundles obtained at 25°. (9) Precipitate prepared at 21° showing wide fibrils, compound fibres and tendency to form sheets (9*b*). Acute fibril bends were also seen (9*a*).

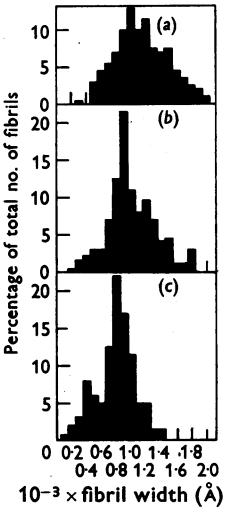

Fig. 7. Distribution of fibril width in precipitates prepared at different pH values: (*a*) pH 6·0; (*b*) pH 7·1; (*c*) pH 8·0. C_0, 0·05%; temp., 25°; *I* 0·23. The number of fibrils measured was 284, 190 and 146 respectively. The data were obtained from the series of electron micrographs of which Plates 1 and 2 (5–8) gives examples.

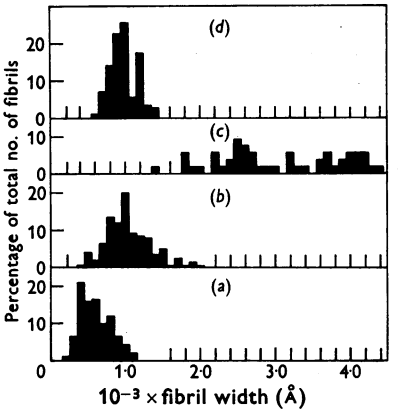

Fig. 8. Distribution of fibril width at different temperatures: (*a*) 37°; (*b*) 25°; (*c*) 21°; (*d*) 25° for 10 min. and then 37°. C_0, 0·05%; temp., 25°; *I* 0·23; pH 7·1. The number of fibrils measured was 140, 195, 51 and 56 respectively. The data were obtained from the series of electron micrographs of which Plate 2 (7–9) gives examples. Expts. (*a*), (*b*) and (*d*) were done on one collagen solution and (*c*) on another.

a dense mat of discrete fibrils mainly of 600–1200 Å width was observed at pH 8 (Plate, 1, photograph 5). As the pH was decreased to pH 7 and 6 the most frequent fibril width increased slightly (Fig. 7). Bundles were observed at pH 6 and 7, and at pH 7 compound fibres, sheets and acute fibril bends were observed (Plate 2, photograph 6). An additional feature at pH 8 was a network of fine filaments which in some areas partially obscured the fibrils.

In the third set of experiments (variation of temperature at pH 7·1, I 0·23) a dense mat of discrete fibrils was observed at 37° (Plate 2, photograph 7). The majority of the fibrils had 640 Å striations but the thinner one showed 220 Å periodicity. As the temperature was reduced the most frequent fibril width and the range of fibril width increased (Fig. 8). Bundles were observed in the 25° precipitate (Plate 2, photograph 8), whereas in the 21° precipitate compound fibres (Plate 2, photograph 9*b*), sheets and acute fibril bends (Plate 2, photograph 9*a*) were seen. Since the 21° experiment was done on a different collagen solution from that used for the 25° and 37° experiments the results are not strictly comparable, but the large difference in fibril width between the 25° and 21° precipitates is probably significant.

Thus in the range of conditions used decrease of temperature and pH and increase of ionic strength tend to increase the most frequent fibril width, increase the range of fibril width and displace it to higher values and increase the extent of lateral aggregation. The results of three experiments (Table 5) done under identical conditions on different preparations of collagen solution indicate the extent to which the morphology of the precipitates is reproducible.

Included in Table 5 are values of E_∞, the final extinction of collagen gels prepared simultaneously with the electron-microscope experiments from the same collagen solutions under identical experimental conditions. It has already been noted that variation of E_∞ with experimental conditions must be due to variations of the morphology of the precipitate, and Table 5 shows that E_∞ increases as the fibril width and extent of lateral aggregation increase. For certain experiments (Table 5) rate of precipitation curves were determined which correspond to the electron-microscope results and are those shown in Figs. 5*a* and 9.

Effect of temperature on the two stages of precipitation

The shape of the precipitation curves suggests that precipitation can be divided into two stages, the first represented by the lag period and the second by the sigmoid portion of the curve. It was of interest therefore to see how each of these steps responded to change of experimental conditions. In order to avoid the necessity for stirring the reaction mixture, temperature was chosen as the experimental variable.

Two identical samples of collagen solution were allowed to precipitate in the usual way, one at 25° (Fig. 9, curve 1), the other at 37° (Fig. 9, curve 2). As in other experiments, increasing the temperature accelerated precipitation and lowered E_∞. The corresponding electron micrographs are given in Plate 2, photographs 8 and 7, and the fibril-width distributions in Fig. 8. These have already been described and we need only reiterate the correlation between E_∞ and fibril width. A third reaction mixture was kept at 25° until the end of the lag period and its temperature then raised as quickly as possible to 37° (Fig. 9, curve 3). Thus although

Table 5. *Summary of electron-microscope data*

Each set of data was obtained with a different preparation of collagen solution. The fibril-width measurements are taken from Figs. 6–8.

pH	I	Temp.	Appearance under low power	Most frequent fibril width (Å)	Range of fibril width (Å)	Lateral aggregation (increasing extent) → Bundles	Compound fibres	Sheets	E_∞
7·1	0·13	25°	Fine	900	200–1700	±	–	–	0·46*
7·1	0·23	25	Coarse	1600	700–2800	+ + +	–	–	1·08*†
7·1	0·31	25	Coarse	2500	800–4100	+ +	–	–	1·15*
6·0	0·23	25	Coarse	1100	500–2000	+ +	–	–	0·74
7·1	0·23	25	Coarse	900	200–1800	+	+	+	0·69†
8·0	0·23	25	Fine	800	100–1400	±	–	–	0·63
7·1	0·23	25	Coarse	1000	400–2000	+ + +	–	–	0·90*†
7·1	0·23	37	Fine	500	200–1200	–	–	–	0·62*
7·1	0·23	21	Coarse	2500	1800–4800	–	+ +	+	—

* Precipitation curves determined on the same collagen solutions are shown in Figs. 5*a* and 9.

† These results were obtained under identical experimental conditions with three different preparations of collagen solution.

raising the temperature to 37° after the lag period accelerated subsequent precipitation, the value of E_∞ was identical with that observed with the temperature at 25° for the whole time. The corresponding electron micrographs and the fibril-width distribution (Fig. 8) were almost indistinguishable from the data obtained with the temperature at 25° throughout precipitation. The results of these experiments are summarized in Table 6. It appears therefore that under these conditions, at least, both sections of the overall precipitation are accelerated by increasing the temperature but that fibril width and hence E_∞ are determined during the lag period.

DISCUSSION

The electron-microscope approach, notably in the hands of Schmitt and co-workers (Schmitt, Gross & Highberger, 1955*a*, *b*; Gross, 1956; Schmitt, 1959), has led to the concept of collagen precipitation *in vitro* as the aggregation of rod-like collagen particles, whose presence in collagen solutions has been demonstrated (Boedtker & Doty, 1956; Doty & Nishihara, 1958; Hall, 1956; Hall & Doty, 1958). In spite of the wide variation of rate of formation, ultimate fibril width and extent of aggregation, all the precipitates observed in the present study consisted, almost entirely, of fibrils bearing striations with 640 Å periodicity. In all cases therefore the soluble-collagen particles must have aggregated in essentially the same manner; probably in the same manner as in the formation of collagen fibrils *in vivo*.

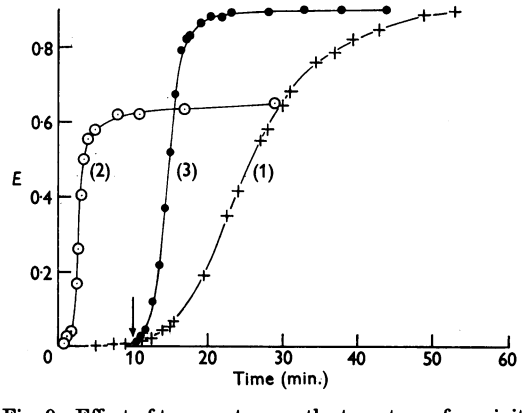

Fig. 9. Effect of temperature on the two steps of precipitation: (1) 25° throughout precipitation; (2) 37° throughout precipitation; (3) 25° during lag period, 37° after lag period. The arrow marks the end of the lag period. C_0, 0·05%; temp., 25°; I 0·23; pH 7·1.

Boedtker & Doty (1956) presented evidence, however, for the presence of aggregated material in some of their solutions, and Mathews, Kulonen & Dorfman (1954) suggested that reversible systems of aggregates occur in collagen solutions. Some of the collagen particles present in our solutions may not therefore have been discrete collagen molecules but molecular aggregates. The presence of aggregates in the cold collagen solutions used in the present work and their slow breakdown when the solutions were brought to 25° or were agitated mechanically might account for the change in viscosity and accompanying change of rate of precipitation (Fig. 3). Differences of rate of precipitation between different preparations of collagen solution and between samples of the same collagen solution of different ages might also be due to differences of extent of molecular aggregation.

Different methods of preparing collagen solutions may well result in starting material, for precipitation experiments, of different degrees of molecular aggregation, and it would not be surprising therefore if precipitation curves determined by Gross & Kirk's or Bensusan & Hoyt's procedures, which involve prior dialysis of the collagen against neutral buffers, differed in detail from

Table 6. *The two steps of precipitation*

The three experiments were done on the same collagen solution. Experimental conditions: pH 7·1; I 0·23; C_0, 0·05%; 25°. The distributions of fibril width are shown in Figs. 8*a*, *b*, *d*. They were obtained from the series of electron micrographs of which Plate 2, photographs 7 and 8 are examples. Values of E_∞, lag period ($t_{0·005}$) and half-growth time ($t_{0·5}$) are taken from the precipitation curves in Fig. 9. For definitions of E_∞, $t_{0·005}$ and $t_{0·5}$ see text.

Temp.	Appearance under low power	Most frequent fibril width (Å)	Range of fibril width (Å)	Lateral aggregation: Bundles	Lateral aggregation: Compound fibres	Lateral aggregation: Sheets	E_∞	$t_{0·005}$	$t_{0·5}$
25° throughout both steps	Coarse	1000	400–2000	+ + +	–	–	0·90	9	16
37 throughout both steps	Fine	500	200–1200	–	–	–	0·62	1	2
25 for first step (10 min.), 37 for second step (remainder)	Coarse	1000	600–1400	+ + +	–	–	0·90	—	6

curves determined in the manner described in this paper. The nature of the molecular aggregates present in solution is unknown but the occurrence of non-striated background filaments in some of the electron micrographs suggests that they may be filamentous in nature (Reed, 1957).

Further information on the mechanism of fibril formation results from correlation of the kinetic and electron-microscope approaches to the problem. In order that the results of the morphological studies should be comparable with those of the rate studies it was necessary to prepare the samples for examination in the electron microscope with the minimum of mechanical disturbance, for, as M. K. Keech (in preparation) has shown, the collagen gels are extremely sensitive to even mild mechanical stress. The electron micrographs indicate that, by replicating the undisturbed precipitate, the integrity of the individual fibrils was, as far as we can tell, maintained. In comparable experiments the final extinction of the collagen gel (E_∞) is correlated with the width of the fibrils, as seen in the electron microscope, and it is reasonable to assume that in other rate experiments for which we do not have comparable electron-microscope data, change of E_∞ is an indication of change of fibril width. Although precautions were taken to make the kinetic and electron-microscope results comparable the latter technique incurs the additional drying of the gel. It is possible that lateral aggregation of the fibrils is a result of the quite large forces which are well known to develop during such drying (see, for example, Anderson, 1956). We cannot say therefore whether or not lateral aggregates of fibrils are present in the gel state and in the following discussion attention is focused on fibril width. However, whether aggregation arises during precipitation or on drying, it was more evident in some precipitates than in others, being most pronounced when the most frequent fibril width was greater than 1000 Å.

When precipitation was accelerated by increasing the temperature, by lowering the ionic strength or by increasing pH from 7 to 8 (Figs. 4 and 5; Tables 3 and 4), the ultimate fibril width was decreased (Table 5). On the other hand when precipitation was accelerated by decreasing the pH from 7 to 6 (Fig. 5 and Table 3) the ultimate fibril width was increased (Table 5). When precipitation was accelerated by increasing C_0 (Fig. 4 and Table 2) the value of E_∞/C_0 remained approximately constant, which suggests that fibril width does not vary much with collagen concentration. Thus there is no simple relationship between rate of precipitation and fibril width. The reason for this becomes clearer when fibril formation is considered as aggregation of elongated particles (discrete rod-like molecules or long aggregates), for it would appear that there are two principal factors determining fibril width. First, fibril width will be partly determined by the number of fibrils: the larger the number of fibrils formed from a given amount of soluble collagen the smaller will be each fibril, and this might reasonably be expected to be correlated with the rate of fibril formation. The second factor arises from the way in which the particles aggregate. According to the ideas put forward by Schmitt and co-workers (Schmitt *et al.* 1955*a*, *b*; Gross, 1956; Schmitt, 1959), they aggregate with their long axes parallel to the axis of the ultimate fibril. They may do this with varying degree of overlap or relative proportion of side-by-side and end-to-end aggregation, which may depend on experimental conditions. The greater the degree of overlap or the greater the proportion of side-by-side aggregation the wider (and shorter) will the resulting fibril tend to be. The observed fibril width will be determined by both these factors, which need not necessarily act in the same direction, although both must be determined by the same interparticle forces.

The way in which rate of precipitation and E_∞ vary with temperature, collagen concentration and ionic strength (Figs. 4 and 5; Tables 2–4) is qualitatively similar to that observed by Bensusan & Hoyt (1958) and Gross & Kirk (1958). The retarding effect of increasing salt concentration at pH 7 and 8 suggests that the rate or manner (or both) of aggregation of the soluble collagen particles is controlled to a significant degree by electrostatic interaction between them. At pH 6, however, there appears to be a salting-out effect at high ionic strength in so far as precipitation at I 0·31 is faster than at I 0·23 (Table 3). A further indication of the importance of electrostatic interaction is provided by the variation of rate of precipitation with pH (Fig. 5 and Table 3). The acceleration resulting from decreasing the pH from 7·5 to 6·0 is consistent with a reduction in the net charge on the collagen as the isoelectric point is approached. The increase of rate on further removal of pH from the isoelectric point (from pH 7·5 to 8·0) is not, however, consistent with this simple electrostatic mechanism. Gross & Kirk (1958), also using phosphate buffers, observed the same acceleration at high pH when precipitation was slowed down to a measurable rate by adding low concentrations of urea. It is possible that change, with pH, of the binding of phosphate ions to the collagen is responsible for the increase of rate on going from pH 7·5 to 8·0. No optimum pH, such as that observed by Bensusan & Hoyt (1958), was observed in the range of pH covered. The ionic strength (0·23) was, however, higher than that (0·105) used by Bensusan & Hoyt, and it is known that the isoelectric point of collagen is strongly dependent on ionic strength

(Jackson & Neuberger, 1957). Moreover, Bensusan & Hoyt (1958) used a different buffer system and, as they point out, different anions can exert different effects on the rate of precipitation. The variation of fibril width with pH is similar to that observed by Vanamee & Porter (1951), although it should be noted that they used a different buffer system.

The shape of the precipitation curves suggests, as pointed out by Bensusan & Hoyt (1958), that precipitation occurs in two effectively consecutive steps, one occupying the lag period and the other represented by the sigmoid precipitation curve. Our results show that both steps are accelerated by increasing the temperature. Moreover, they show that fibril width in this closed system is determined during the lag period, i.e. during the first step, and is not affected by a subsequent change of rate of precipitation, at least when temperature is the experimental variable. It would appear therefore that the ultimate fibril width is decided before any appreciable precipitation has taken place. This can only mean that all or most of the fibrils are formed during the lag period. Jackson (1955), in her quantitative study of the formation of collagen fibrils in tissue cultures of embryonic avian tendon, noted that at any stage in their formation there was only a small variation of fibril width in any one bundle of fibrils and suggested that all the fibrils in a bundle must therefore have formed almost simultaneously at a very early stage. These results thus support the view that fibrils form *in vivo* by a similar mechanism to the formation of fibrils *in vitro*.

The width of collagen fibrils observed *in vivo* varies widely from tissue to tissue (Banfield, 1954; Gross, 1948, 1950; Schwartz, 1957). The results suggest that differences of the pH, ionic strength and temperature in the tissue at the time of fibril formation might be significant factors determining this variation.

Opacity measurements (Shulman & Ferry, 1950; Edsall & Lever, 1951) and electron microscopy (Hawn & Porter, 1947; Porter & Hawn, 1949) have been used to study the conversion of fibrinogen into fibrin clots, a system somewhat similar to the precipitation of collagen (Vanamee & Porter, 1951; Schmitt *et al.* 1955*a*; Schmitt, 1957). As in the collagen system, coarseness of the precipitate or clot (wide fibrils and high degree of lateral aggregation of fibrils) is associated with high opacity, and vice versa, and there is no simple correlation of these properties with rate of precipitation.

In view of the fact that the fibrinogen–fibrin conversion involves an enzymic step, however, detailed comparison of the two systems at this stage would not be profitable, for it seems unlikely that a similar enzymic step occurs during the collagen precipitation *in vitro* discussed above.

SUMMARY

1. The precipitation of collagen fibrils in solutions of acid-soluble collagen extracted from calf dermis has been followed turbidimetrically under a range of experimental conditions and the final precipitates have been examined by electron microscopy.

2. All the precipitates consisted of fibrils bearing 640 Å striations and in each precipitate the distribution of fibril width was fairly narrow. Lateral aggregation of the fibrils was most pronounced in those precipitates containing the widest fibrils.

3. When the rate of precipitation was increased by increasing the temperature or lowering the ionic strength, fibril width decreased, but when the rate was increased by reducing the pH, fibril width increased. The variation of rate of precipitation with ionic strength and pH indicates that it is controlled to a significant degree by electrostatic interaction between the soluble collagen particles.

4. All precipitation curves showed a lag period, followed by a sigmoid growth curve. Both phases were accelerated by increase of temperature but the width of the ultimate fibrils was affected only if the temperature was increased during the lag period. It is concluded that most of the fibrils were formed during the lag period.

5. These observations are discussed in relation to the development of collagen fibrils *in vivo*.

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The Formation of Fibrils from Collagen Solutions

2. A MECHANISM OF COLLAGEN-FIBRIL FORMATION

BY G. C. WOOD
Nuffield Gerontological Research Unit, Department of Medicine, University of Leeds

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In the preceding paper (Wood & Keech, 1960) evidence was adduced for the hypothesis that precipitation of collagen fibrils from collagen solutions occurs in two steps, the first occurring during the lag period, the second being represented by the sigmoid precipitation curve. This suggestion has also been put forward by Bensusan & Hoyt (1958). The ultimate fibril width appears to be determined during the first step. It is the purpose of the present paper to see how the experimental results may be interpreted quantitatively in terms of a two-step mechanism.

Freshly prepared collagen precipitates can be redissolved by altering the pH or temperature and then reprecipitated by returning these variables to their original values (Jackson, 1957; Gross, 1958). It seems reasonable therefore to regard the formation of collagen fibrils as precipitation of the protein, in an ordered form, from solution in which it is supersaturated under the particular conditions of pH, ionic strength, temperature, etc. One might expect therefore some similarity between the precipitation of collagen and the precipitation of sparingly soluble inorganic salts, in crystalline form, from supersaturated solution.

The kinetics of the latter process has been widely studied and in many cases it has been recognized that the process takes place in two steps: (*a*) aggregation of the particles in solution to form nuclei, i.e. clusters of particles of just sufficient size to be stable as a separate phase: (*b*) growth of these nuclei, by accretion of soluble material, to