THE FORMATION OF FIBRILS FROM COLLAGEN SOLUTIONS

IV. Effect of Mucopolysaccharides and Nucleic Acids: An Electron Microscope Study

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ABSTRACT

The kinetics of collagen reprecipitation from solutions of salt-extracted calf dermis in the presence of small amounts of mucopolysaccharide and nucleic acids (0.005 per cent in the final reaction mixture) has been reported by Wood (1960). The present paper is a parallel study using the same materials, and describes the electron microscopic (EM) morphology of the collagen precipitates replicated after 24 hours at room temperature. Satisfactory, uncontaminated EM preparations were obtained which showed that all the deposits were fibrous and bore the 640 A cross-banding characteristic of collagen except some narrow, background fibrils 200 to 1000 A wide precipitated in the presence of heparin. These exhibited fine striations about 220 A apart. Chondroitin sulfate greatly increased the rate of precipitation to give a deposit of low optical density consisting of narrow, rigid, discrete fibrils resembling fresh dermis. In contrast, heparin prevented macroscopic gelation, delayed precipitation, and only produced a scanty deposit of abnormal, short, wide, striated tactoids and compound fibers of varying length. The control preparations and the deposits formed in the presence of hyaluronic acid were intermediate between these two extremes. Delayed precipitation was associated with a coarser deposit and aggregation of the fibrils. A duplicate series of deposits precipitated in the presence of RNA and DNA, together with their controls, were examined after ½, 1, 1½, 3, 9, and 24 hours. One set employed an acetic extract of whole calf dermis and the other salt-extracted dermis. The presence of 0.005 per cent DNA in the reaction mixture markedly delayed collagen precipitation with the slow formation of abnormal, short, wide tactoids and compound fibers. RNA also interfered with the quantity and quality of the deposits which contained far less collagen resembling unfixed, normal, adult human dermis, than the controls at the corresponding time intervals. Comparison of the experiments employing whole calf dermis with those employing the salt-extracted material demonstrated that at every time interval in all the experiments the deposits were retarded when salt-extracted dermis was used. This suggests that the salt-soluble components of the dermis play a part in fiber formation.

Previous work in this series of experiments (1–3) described the kinetics of collagen precipitation from salt-extracted calf dermis based on a systematic study of the effect of experimental conditions on both the rate of fibril formation and the morphology as seen under the electron microscope. Samples of the identical solutions used for the rate studies revealed that factors which speeded up the precipitation of neutral salt collagen (increased temperature; decreased ionic strength)

produced a low optical density and thinner fibrils, whereas the reverse obtained when precipitation was delayed (decreased temperature; increased ionic strength). The present paper reports the morphological effect of mucopolysaccharides on fibril formation and parallels the physical chemical results reported by Wood (3). In addition, it describes the effect of nucleic acids on collagen as precipitated from extracts of whole calf dermis as well as from the salt-extracted material.

MATERIALS AND METHODS

Part I: Effect of Mucopolysaccharides

Collagen Solutions: These were prepared from the same salt-extracted preparation used previously and designated Dermis X (1). Briefly, fresh calf dermis was defatted, dehaired, homogenized, exhaustively extracted with salt at pH 7.0, washed and freeze-dried. Samples of this purified dermis were then extracted with 0.5 M acetic acid at 0 to 4°C., filtered, and the clear filtrate dialyzed against 4 changes of 0.1 M NaCl containing 0.05 M acetate buffer, pH 4.2. The resulting viscous solution was centrifuged and the collagen stored at 0–4°C. after the concentration had been adjusted to 0.1 per cent by dilution with cold 0.1 M NaCl. Assuming that the hydroxyproline content of collagen is 8.2 gm. of amino acid N/100 gm. of N, the hydroxyproline content of this solution (Dermis X) indicated that about 93 per cent of the dissolved protein was collagen. This collagen was completely precipitated under the experimental conditions employed in this study.

Mucopolysaccharides: Five samples of chondroitin sulfate (CSA *a*, *b*, and *c* kindly supplied by Professor K. Meyer, and two commercial preparations obtained from cattle trachea by Evans Medical Supplies and L. Light & Co.); heparin (British Drug Houses); purified hyaluronic acid (L. Light & Co.); and two preparations of bovine intervertebral disc (F1 and F2) obtained from Dr. P. F. Lloyd. Ionophoresis indicated that F1 consisted predominantly of keratosulfate and F2 of hyaluronic acid. All these substances were made up as 0.01 per cent solutions in phosphate buffer pH 7.1.

Examination of the Substances Alone: Drops of the solutions of collagen and mucopolysaccharides were placed on collodion-covered grids (without grinding), drained with filter paper, allowed to dry, washed with distilled water, showed with chromium, and examined in a Siemens electron microscope, type UM 60 C.

Experimental Method: After the collagen solution had attained room temperature (23 to 25°C.) by standing on the bench for exactly 1½ hours, and the other materials for a minimum of 10 minutes, 1.7 ml. of the latter were added to 1.5 ml. of the collagen solution and mixed by inverting the tube 3 times. Approximately the same amount of each sample was immediately transferred by micropipette to duplicate slides, spread to make pools 1 to 2 mm. deep and placed in petri dishes. Extra buffer in tinfoil cups was put inside each dish to minimize evaporation of water from the reaction mixtures. Precautions were taken that these mixtures remain undisturbed during the experimental time intervals by protection from movement, draughts, etc.

After 24 hours at 23 to 25°C., the slides were dried in a stream of air at room temperature from a hair dryer fixed 18 inches from the slides; this procedure took 10 to 15 minutes. Each sample was gently washed with distilled water to remove buffer salts, dried again in the same way, shadowed with gold-palladium at an angle of 5° and covered with a thin film of collodion. After this film was scored into small squares, and immersed in trypsin solution overnight at room temperature to remove dermal components, the loosened squares were peeled from the slide, washed in a dish of water and picked up on EM grids. The final preparation thus consisted of a metal-shadowed replica of the surface of the undisturbed, washed and dried precipitate. All the specimens were examined in the EM, large areas of several grids from each sample being carefully scrutinized and special attention paid to the relative quantities of the various structures seen. Over 150 electron micrographs of representative areas were taken, including samples of the various substances alone. The test tubes containing the remainder were examined macroscopically at intervals.

Controls were performed using phosphate buffer without any added substance. The reaction mixture had a collagen concentration of 0.05 per cent, a phosphate concentration of 0.04 M, an ionic strength (μ) of 0.23, and contained 0.005 per cent mucopolysaccharides. The experiments were performed in two batches. The first batch included the control and all the mucopolysaccharides listed above; the second consisted of repeat control, CSA *a* and CSA *b* experiments, as well as a more dilute solution of heparin (0.0005 per cent). No antibacterial agents were used, but by employing only freshly made collagen and buffer solutions bacterial contamination was avoided.

Part II: Effect of Nucleic Acids

Collagen Solutions: Samples of the homogenized dermis from the same calf skin were treated in two ways: (*1*) extracted with 0.5 M acetic acid and the resulting solution dialyzed against 0.1 M NaCl to give

a clear, viscous solution with a collagen content of about 0.1 per cent; (*2*) exhaustively extracted with NaCl as follows: 50 gm. dermis was stirred gently with 650 ml. of 0.2 M Nacl, pH 7.0, for 16 hours. Undissolved material was separated by centrifuging and extracted again for 16 hours with 500 ml. of 0.2 M NaCl, pH 7.0, and once with saturated NaCl. The residue was washed free of salt and then treated as under (1). The latter preparation was very similar to the Dermis X employed in Part I except that only 50 to 60 per cent of the collagen was precipitated under the experimental conditions used. For brevity, the extract from whole calf dermis will be referred to as Dermis Y_1 and from salt-extracted dermis as Dermis Y_2.

Nucleic Acids: Samples of deoxyribonucleic acid (DNA_3, 0.04 per cent in water) and ribonucleic acid (RNA_3, 0.05 per cent in 0.1 M NaCl) were obtained from G. C. Wood.

Buffer: Phosphate buffer, pH 7, μ = 0.2.

Experimental Method: This was the same as that employed in Part I except that: (*1*) an equal volume of buffer alone or of buffer containing 0.01 per cent of RNA or DNA was added to the collagen solutions obtained from Dermis Y_1 or Y_2; (*2*) the Petri dishes containing the slides were kept at bench temperature (20 to 23°C.), duplicate samples being dried at ½, 1, 1½, 3, 9 and 24 hours. A minimum of 2 grids were carefully scrutinized at a magnification of 10,000 from each set of experiments at each time interval; *i.e.*, 2 × 36 grids. 25 squares could be visualized on each grid, and about 25 fields were needed to cover each square. Thus, as each grid contained 625 fields, a total of 72 × 625, or 45,000, fields were examined in Part II of the present study. 640 electron micrographs of representative areas were taken, including samples of the various substances alone.

Terminology: Lateral aggregation of the collagen fibrils occurred as previously reported (1). *Bundles* composed of fibrils mainly 1000 to 1400 A in width (Fig. 4) were found in all the experiments employing mucopolysaccharides except heparin. Lateral aggregation of the fibrils in one plane, their cross-striations in register, but with partial loss of individual outlines to form flat *sheets* was seen in the controls (Fig. 3). Where these sheets occurred in long, wide bands, they were termed *compound fibers*. Compound fibers could either be formed of wide individual fibrils 2000 to 4000 A in diameter (control experiments, Fig. 3); of fibrils of moderate width (1000 to 2000 A), as in the DNA experiments (Figs. 13 and 14), or of narrower tactoids and fibrils 1000 to 1400 A wide (heparin experiments, Fig. 5). As these compound fibers were frequently very thick and up to 16,000 A in diameter, only examples of small or moderate size could be adequately micrographed.

Collagen Grading: The collagen fibrils of normal adult skin are mainly 1000 to 1400 A in width, rigid and of indefinite length. Narrow fibrils and visualization of the tapered fibril ends characterize young skin or actively growing tissue (4–6). The presence of acute bends is evidence of flaccidity of the fibrils as the suspension settles on the grid during drying (7). As all the replicated precipitates described here were satisfactory, uncontaminated preparations consisting almost entirely of collagen bearing 640 A striations, with hardly any debris and no elastin-like structures, amorphous material or buffer salts, these characteristics at each time interval could be easily established. Although the deposits were reprecipitated

TABLE I

Effect of Mucopolysaccharides on Collagen Fibril Formation

Analysis of fibrous deposit after 24 hours at 23 to 25°C., using Dermis X and phosphate buffer pH7, μ = 0.2. The fibril width was measured with a scaled Leitz eyepiece on 126 photographic plates. Normal (dermal) fibril width is 1000 to 1400 A. All fibers exhibited 640 A cross-banding except in the heparin experiment, in which fibrils <1,000 A had 220 A striations.

	Control	CSA	K (F1)	PHA	IHA (F2)	H
Basic fibril width (A)		600				
<1,000	—	+	occ.	occ.	—	+++
1000–1400	occ.	++	++	+++	+	+
2000–4000	++	—	—	—	+	—
Compound fibers	++	—	—	—	—	++
Fibril length	Long	Long	Long	Long	Long	Short
Collagen grading	1	3	3	2	1	1

occ., occasional; CSA, 5 samples of chrondroitin sulfate; K, keratosulfate (F1); PHA, purified hyaluronic acid; IHA, impure hyaluronic acid (F2); H, heparin 0.005 per cent in the final mixture. Wood's histograms of fibril width (Fig. 16) were based on the EM photographic plates of the CSA experiments.

TABLE II

Effect of Nucleic Acids on Collagen Fibril Formation

All experiments at bench temperature, 20 to 23°C.; 640 A cross-banding throughout

		Control		RNA		DNA	
		Dermis Y_1	Dermis Y_2	Dermis Y_1	Dermis Y_2	Dermis Y_1	Dermis Y_2
EM replicas × 300	At 24 hours	Coarse network of long fibers	Less dense network of long fibers	Less dense network of wider fibers	Network of wider fibers	Short, wide tactoids and fibers of varying length	Fewer short wide fibers and slender background tactoids
EM replicas × 10,000	Time (hr.)	½ 24	½ 24	½ 24	½ 24	At ½ hr., heavy fibrous background and NSF. At 1 hr., scanty, short, striated tactoids and fibrils 1000–4000 A wide; as incubation progressed, the striated fibrils became more numerous, wider and longer until at 24 hr. a heavy deposit of compound fibers up to 15,000 A wide and a background of narrow striated fibrils 600–1000 A wide	Delayed sequence as compared with Dermis Y_1 At ½ hr., no fibrous background and no fibrils of any kind At 1½ hr. onward, background material similar to Dermis Y_1 At 9 hr., very scanty striated tactoids and compound fibers At 24 hr., moderate deposit of compound fibers 2,000–15,000 A wide. Some slender 1000 A tactoids and scanty NSF
	M.N. coll.	85% 95%	70% 85%	70% 70%	50% 50%		
	Coll. grad.	2 3	2 3	1 1	1 1		
		Increasing bundling of 1000–1400 A fibrils and appearance of wider fibrils measuring 1600–2000 A as incubation progressed; no NSF; dermis Y_2 similar sequence to dermis Y_1 only less M.N. coll. and fewer wider fibrils		1000–1400 A fibrils which were gradually replaced by wider fibrils measuring 2000–4000 A; fibril bends; no NSF; no fibrous background	Similar to Dermis Y_1 but less M.N. coll.; Progress slower and fewer wide fibrils; long TE; fibril bends; scanty NSF; fine fibrous background		

Dermis Y_1 and Y_2, acetic acid extracts of whole dermis and salt-extracted dermis, respectively, prepared from the same calf skin. Control, Dermis + buffer pH 7.0, + H_2O; RNA, Dermis + buffer + RNA_3; DNA, Dermis + buffer, + DNA_3.

M.N. coll., collagen resembling unfixed, normal, adult human dermis; Coll. grad., collagen grading; NSF, nonstriated background filaments; TE, tapered fibril ends.

(dermal) collagen and not fresh tissue, the following morphological separation into grades proved valuable, especially for denoting the stage of "advancement" reached by each deposit at each serial time interval employed in Part II (Tables I and II).

Grade 1: Apparently undivided fibrils 2000 to 4000 A in width, occurring singly or in bundles. A few 1000 to 1400 A fibrils. Frequent acute fibril bends and tapered ends.

Grade 2: Some 2000 to 4000 A fibrils, but mainly fibrils 1000 to 1400 A wide, occurring singly or in bundles. A few tapered ends.

Grade 3: Long, rigid fibrils 1000 to 1400 A wide, occurring singly or in bundles. Resembles collagen from unfixed, normal, adult human dermis.

It should be emphasized that this grading is valid for comparative purposes within a particular set of experiments using the *same* collagen extract, but, although the principle still holds good, the actual appearance in each grade might differ slightly if another solution of collagen were employed.

Measurement of Fibril Width: In conformity with present terminology, the term "fibril" denotes an apparently single structure at the magnification used. Fibril width was measured on the EM 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

Part I: Effect of Mucopolysaccharides (Table I)

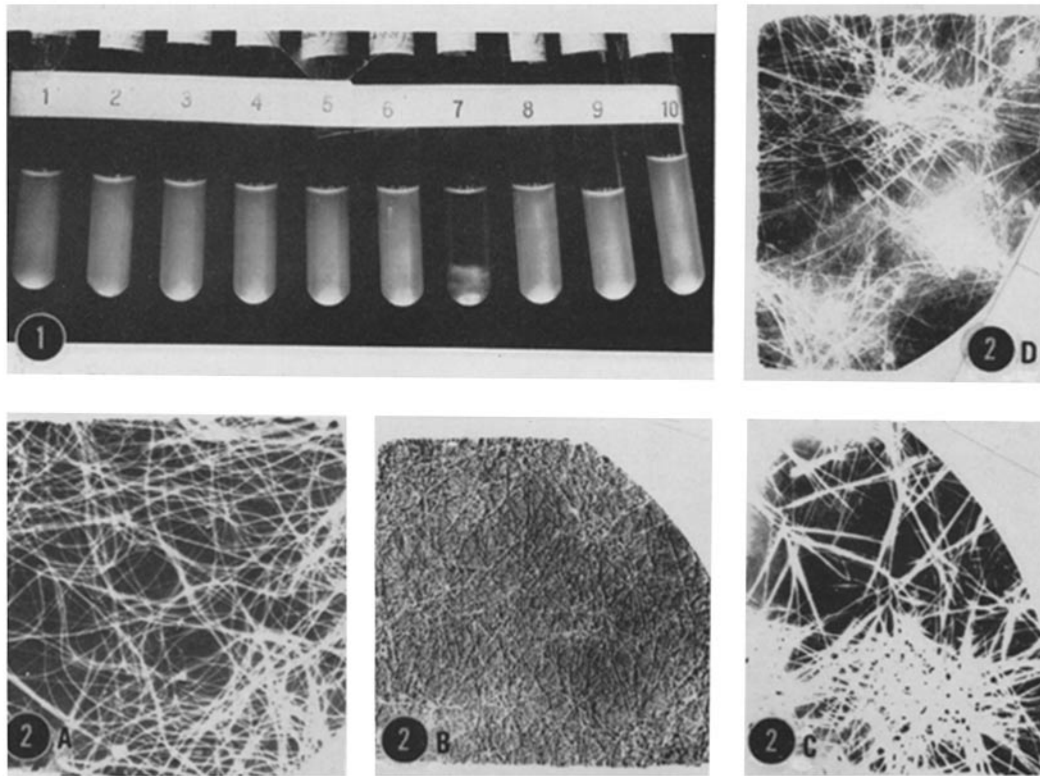
Macroscopic Findings: The remainder of the reaction mixture in the test tubes quickly forms a homogeneous opaque gel as shown by Wood (3), and this remains unchanged throughout the 24 hours (Fig. 1). The only exception is the experiment employing the more concentrated (0.005 per cent) solution of heparin, in which only a very faint opacity appears in the upper half of the tube. The 0.0005 per cent solution fails to prevent the formation of the usual gel precipitate.

Electron Microscope Findings

Examination of the deposits under the light microscope (× 60) and with the EM at low power (× 300) reveals that all the experiments employing CSA give a dense mat of fine fibers (Fig. 2*B*), whereas the control (Fig. 2*A*) and hyaluronic acid preparations present a coarser precipitate of wider fibers. The 0.005 per cent solution of heparin produces a totally different picture (Figs. 2*C* and *D*). A scanty deposit is situated round the edges of the preparations on the glass slides and contains star-shaped collections of very wide, relatively short tactoids which appear as refractile, needle-like crystals under the light microscope. Under the EM there are, in addition, some areas consisting of a fibrous network of longer, thinner fibrils exhibiting acute bends and tapered ends. The 0.0005 per cent solution of heparin fails to reproduce this abnormal picture, and gives a coarse network of wide fibers similar to the control.

High Power Examination (× 10,000): The collagen solution alone gives an identical picture to that described previously (1), and consists of nonstriated filaments of varying size, mainly 1,000 A in diameter, some with tapered ends. No striated collagen fibrils are seen. Samples of the mucopolysaccharides alone (0.01 per cent solutions) give no distinctive morphology apart from scanty, granular amorphous material. *Control Experiments (Fig. 3). Collagen Grade 1:* Collagen reprecipitated by the addition of phosphate buffer alone presents a similar morphological picture in repeated experiments, with wide fibrils (mainly 2000 to 4000 A in diameter) exhibiting a marked tendency to aggregate laterally to form the sheets and compound fibers described under *Terminology*. There are occasional fibrils 1200 to 1400 A wide (the narrowest seen), a few acute fibril bends and tapered ends, but no background filaments. All the structures bear 640 A cross banding.

Experiments Using Different Samples of CSA (Fig. 4). Collagen Grade 3: In the experiments using the 3 different purified fractions (CSA *a*, *b* and *c*) supplied by Professor K. Meyer, the 24 hour precipitations are essentially similar on repeated occasions. They differ from the controls in consisting of an apparently denser feltwork of narrower striated fibrils arranged singly, in pairs or in bundles. No compound fibers are seen. The dominant fibril width is 1000 A, but there is quite a high proportion of fibrils measuring 500 to 600 A, and frequently 2 are closely adjacent throughout to give a total width of 1000 A. This lateral alignment can usually be distinguished in all the CSA preparations, and sometimes the division of a fiber into its component fibrils can be seen along part of its length. The fibrils are long and rigid, and only occasional tapered ends are visible in the thinner areas of the deposit. Scanty to moderate numbers of nonstriated background filaments are seen throughout,



All photographs (except Figs. 1, 6 and 7) are of replicated specimens shadowed with gold-palladium. The scale marked on the plates represents 1 μ. All striated structures exhibited 640 A cross-banding unless otherwise stated.

FIGURE 1

Macroscopic appearance of the experiments reported in Part I after 24 hours at 23 to 25°C. The usual opaque homogeneous gel of re-precipitated collagen has formed in all tubes except No. 7 which contained heparin (0.005 per cent in the final mixture). Only a faint opacity occurred in the upper half of the tube, but this settled to the lower part during transport for photography.

FIGURES 2*A* to *D*

Lower Power Electron Micrographs. × 600.

A, Control. Coarse meshwork of long, wide fibers.

B, Very dense mat of fine fibers formed in the presence of CSA *a*. All the other samples of CSA gave similar deposits.

C and *D*, the presence of heparin (0.005 per cent in the final mixture) prevented gelation. In some areas of the deposit, star-shaped clusters of short, wide tactoids were seen; in others, networks of long fibers of varying width and length with long tapered ends.

being most pronounced in the experiments using CSA *b*. The filaments vary from examples up to 8000 to 10,000 A long and 200 A wide, which describe a wavy course and have attenuated terminations, to short, linear aggregates of tiny, beadlike particles. These particles can be seen lying free in some areas. The 2 commercial preparations of CSA from cattle trachea (presumed mixtures of CSA *a* and *c*) give very similar, though less dense, precipitates to that obtained from the purified samples.

The histograms (Fig. 16) were constructed by G. C. Wood after measuring the fibril width on the EM photographic plates from these experi-

ments. They demonstrate that there is no significant difference in fibril width in the precipitates formed in the presence of the 5 different samples of CSA. In all cases, the range is 100 to 1500 A, but the most frequent fibril width is 600 to 1000 A. In contrast, the control shows a much wider distribution of fibril width (1300 to 3200 A), the majority measuring 2000 to 2800 A.

Experiments Using F1 (Keratosulfate). Collagen Grade 3: The precipitate is morphologically very similar to that found in the CSA experiments. However, there are some tapered fibril ends and a moderately dense network of background filaments.

Experiments Using Hyaluronic Acid: The purified sample gives a heavy deposit of fibrils 1000 to 1200 A wide occurring singly or in bundles. No compound fibers are seen. Some of the fibrils show the longitudinal division between the two components and there are occasional acute fibril bends, tapered ends and a moderate number of nonstriated background filaments. Collagen Grade 2.

The impure preparation of hyaluronic acid (F2) contains fewer narrower fibrils, some examples 2000 and 3000 A wide, and more acute fibril bends and tapered ends. No background filaments are seen. Collagen Grade 1.

Experiments Using Heparin at 2 Different Concentrations: (*a*) 0.005 per cent concentration in the final mixture (Fig. 5). Collagen Grade 1. This presents quite a different picture from the other experiments. Long and short striated tactoids, and long compound fibers 2000 to 12,000 A wide (see *Terminology*) are seen in all stages of formation. There are occasional acute fibril bends and 640 A cross-banding occurs throughout. The background is also different from any of the other experiments. It consists of a dense feltwork of fibers 200 to 1000 A wide, the majority measuring 200 to 500 A, the narrower examples bearing fine cross-striations one-third the usual spacing, *i.e.*,

FIGURE 3

Replicated control specimen employing Dermis X. This deposit consisted of fibrils mainly 2,000–4,000 A in width, which exhibited a marked tendency to aggregate laterally in one plane, with their cross-striations in register but with partial loss of identity, to form sheets and the wide bands termed compound fibers (collagen grade = 1). × 20,000.

about 220 A. This fibrous meshwork sometimes completely surrounds the larger compound fibers so that, in the surface replicas examined in the EM, a proportion of the wide fibers are partially obscured by these smaller fibrils.

(*b*) 0.0005 per cent concentration in the final mixture. The moderately heavy deposit consists of bundles 2000 to 12,000 A wide, composed of long fibrils 1000 to 1400 A in diameter. There are quite a number of long tapered ends and acute fibril bends. Cross-banding is 640 A, but the fibrous background meshwork characterizing the more concentrated preparation is absent.

Part II: Effect of Nucleic Acids (Table II)

Macroscopic Findings: The reaction mixtures of the control and RNA experiments employing the extract from whole dermis (Y_1) quickly form a solid opaque gel which remains unchanged throughout the 24 hours (Fig. 6). The control experiment using the salt-extracted material (Y_2) gives a similar picture but not until 1½ hours, whereas the ½ hour RNA sample contains an opaque gel which remains unchanged throughout. In contrast, macroscopic gelation is absent in both sets of experiments employing DNA. With Dermis Y_1, a very fine particulate suspension only becomes visible at 3 hours and this is opaque and flocculent by 9 hours. The sequence of events is delayed with Dermis Y_2, no definite suspension being seen until 24 hours, when the fluid is slightly turbid. These findings confirmed the detailed studies on the rate of precipitation reported by Wood (3).

Electron Microscope Findings

Low Power Examination (× *300*)*:* Both control precipitates consist of coarse networks of long fibers of moderate width, whereas both experiments employing RNA contain a higher proportion of wider fibers. In contrast, the two DNA deposits are less profuse and contain some very wide fibers and tactoids of varying length. These are not so numerous with Dermis Y_2, but the background contains many shorter, slender tactoids.

High Power Examination (× *10,000*)*:* Samples of the collagen solutions alone, prepared from Dermis Y_1 and Y_2 (Fig. 7) are similar to Dermis X described in Part I. RNA alone appears as a structureless, bubbly material apparently composed of a thick layer of numerous, very tiny, beadlike particles. DNA alone has a coarser background with superimposed linear aggregations resembling branching "filaments."

Control Experiments Using Dermis Y_1 and Y_2 (Figs. 8 and 9): The findings are summarized in Table II. Although both series of deposits are similar, that formed from the salt-extracted dermis contains less collagen typical of unfixed, normal, adult human skin.

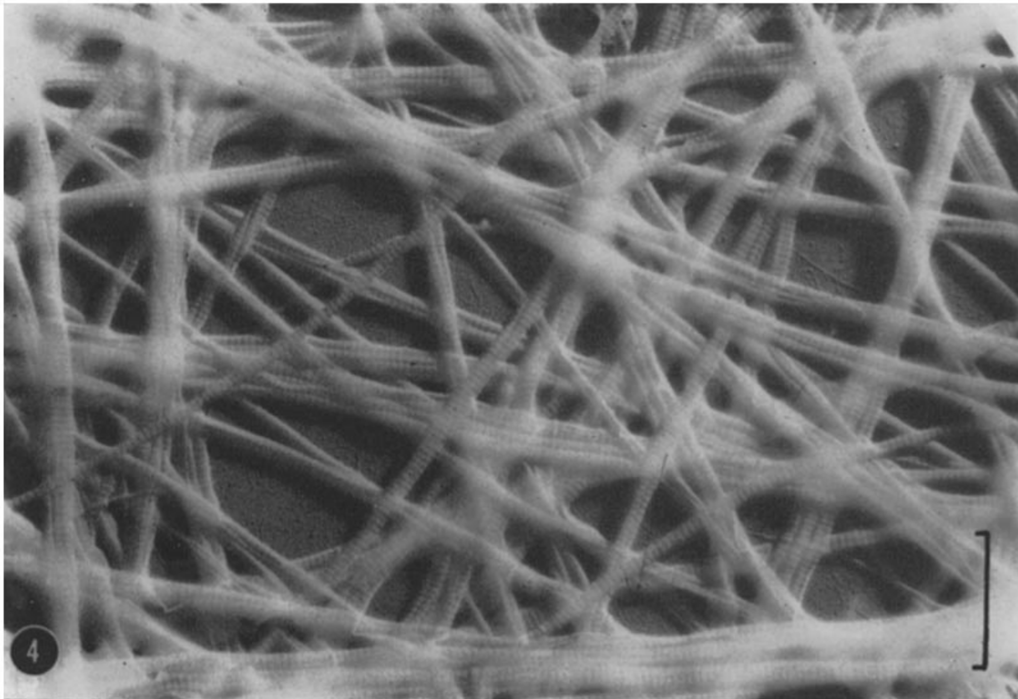
RNA Experiments Using Dermis Y_1 and Y_2: The findings are summarized in Table II. Both collagen solutions give similar precipitates but, again, all the samples from the salt-extracted dermis are less like unfixed normal, adult, human skin. Comparison with the control series reveals that, at each time interval, the presence of RNA significantly alters the picture. There is a smaller

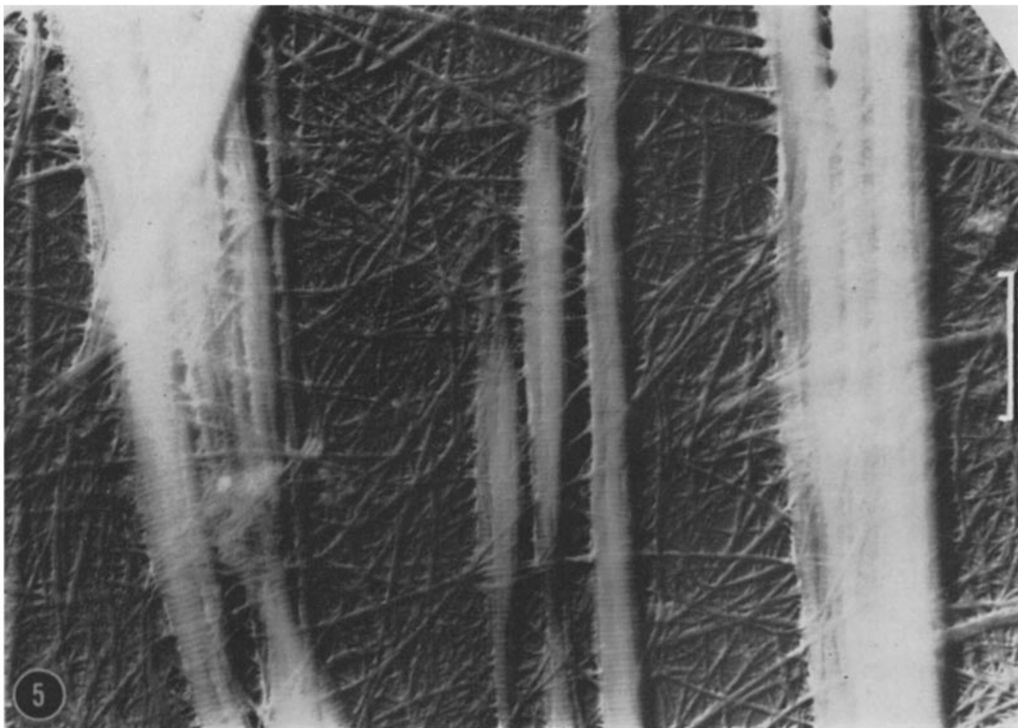
FIGURE 4

Dermis X with purified sample of chondroitin sulfate C. Heavy mat of rigid fibrils 1,000 A in width, occurring singly and in bundles; scanty non-striated background filaments and scattered, bead-like particles (collagen grade = 3). Identical deposits were found with CSA *a* and *b*, and the cattle trachea preparations gave similar, though less dense, precipitates with more background filaments. × 20,000.

FIGURE 5

Dermis X and heparin (0.005 per cent) in the final mixture. The deposit consisted of: (1) narrow, striated tactoids and fibrils aggregated laterally in one plane to form compound fibers. (Much larger ones were seen but proved difficult to photograph adequately); (2) A heavy fibrous background. The majority of these fibrils measured 200 to 1,000 A in width, the narrower examples bearing 220 A cross-bands. × 20,000.





proportion of the adult type of collagen and, unlike the controls, this does not increase with increasing incubation time, while acute fibril bends and long tapered ends are frequently seen.

DNA Experiments Using Dermis Y_1 and Y_2 (Figs. 10 to 15): These differ markedly from the control and RNA experiments, and the development of striated fibrils is greatly delayed in the Y_2 (salt-extracted) material as compared with Dermis Y_1. From a fine fibrous background of nonstriated filaments, scanty slender striated tactoids appear to be formed. These progressively increase in size to become compound fibers which seem cylindrical, and consist of closely packed component fibrils 1000 to 2000 A wide (Figs. 13 and 14). Although very thick ones are seen, only examples of moderate size could be adequately photographed. As the striated component increases in amount, the background material decreases. The dominant length of all the structures seen in all the precipitates is much shorter than in the other 2 series of experiments, and is associated with the lack of gelation illustrated in Fig. 6. It should be stated that 2 to 2½ times more grid area was scanned in all the DNA preparations in order to make absolutely certain that no fibrils were present at the earlier incubation times. This lack of fibrils rendered the collagen grading inapplicable.

DISCUSSION

Although experiments involving the addition of CSA, RNA, DNA and heparin to soluble collagens under varying conditions have been reported, none are strictly comparable with those described in the present study. Schmitt *et al.* (8) reported the immediate production of structureless filaments when yeast RNA and DNA dissolved in 0.05 M citrate, pH 4, were added to ichthyocol filtrate. Gross (9) later reported that typical "segment long spacing" (SLS) could be produced by crude DNA, RNA, heparin and CSA if the latter were added to a cold solution of collagen at neutral pH and the clear solution *dialyzed* against an acid buffer (pH 3 to 5). Fitton Jackson and Randall (10) found that the addition of CSA to solubilized rat tail tendon gave an immediate precipitate consisting of structureless fibrils under the EM. Dialysis against tap water to pH 7 did not alter the picture. However, the addition of sodium acetate in the pH range 4.8 to 7 gave normal fibrils with 640 A banding. I

FIGURE 6

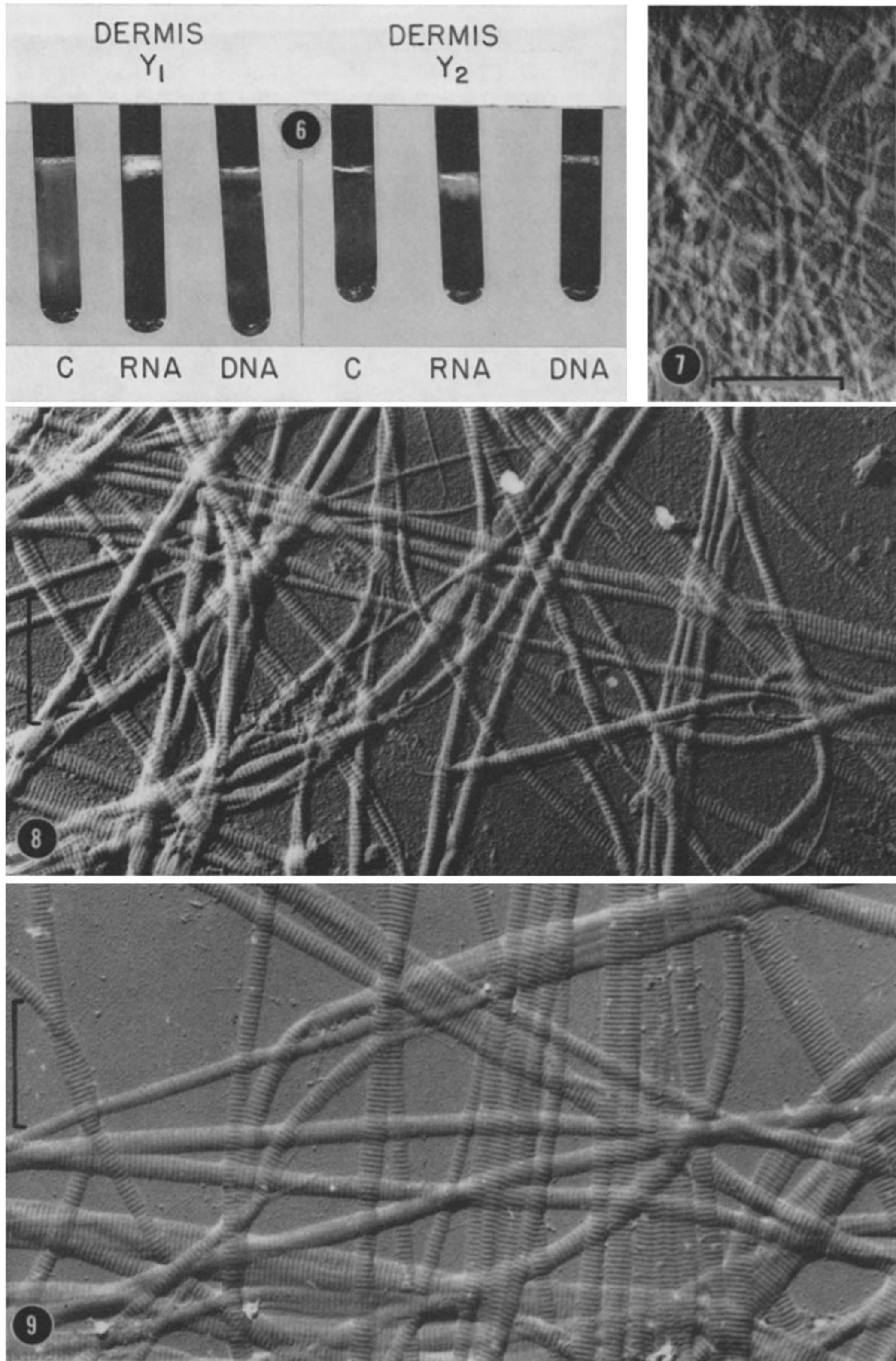
Macroscopic appearance of the experiments reported in Part II after 24 hours at 20–23°C. The control tubes contain an opaque gelatinous precipitate; the RNA tubes a particulate gelatinous precipitate which has risen to the surface following shaking; and the DNA tubes a very slight suspension without gelation. In each experiment the precipitate is less marked using salt-extracted dermis (Y_2) than with the collagen solution from whole calf dermis (Y_1).

FIGURE 7

A drop of the clear, viscous collagen solution obtained from whole calf dermis (Y_1) was air-dried on the microscope grid, washed and shadowed with chromium. EM examination revealed a thick feltwork of filaments: no striated collagen fibrils were seen. The salt-extracted dermis (Y_2) presented a very similar appearance. × 20,000.

FIGURES 8 and 9

Replicated control experiments using the collagen solution from whole dermis (Y_1). At 30 minutes (Fig. 8) the fibrils were narrow with some tapered fibril ends (collagen grade = 2). By 24 hours (Fig. 9) the deposit consisted almost entirely of wider, longer fibrils with very few visible fibril ends (collagen grade = 3). At each time interval the corresponding deposits formed by the salt-extracted material (Y_2) (not illustrated) contained less collagen typical of adult dermis and more tapered fibril ends. × 20,000.


DERMIS
Y1
DERMIS
Y2
C
RNA
DNA
C
RNA
DNA

dialyzed immediately, some of the normal fibrils were converted into the "long spacing" (LS) form. Their report on the immediate precipitate of apparently normal fibrils by CSA in the presence of salt (before dialysis) is the nearest approach to the experiments described in the present investigation. The present study, it should be emphasized, was performed at neutral pH throughout.

All the precipitates described here were fibrous and the structures exhibited 640 A cross-banding. However, in the experiment employing heparin, in addition to the 640 A banding of the wider structures, some of the finer background fibrils bore 220 A striations. As shown in Table I, very small amounts of mucopolysaccharide produced a marked effect on the resulting precipitate. Examination of the dilute solutions of the mucopolysaccharides alone, even at twice the concentration present in the final reaction mixture, showed that they lacked any definite morphology. Thus, their presence could only alter the morphology of the precipitates by affecting the forming collagen to give the very distinctive pictures found. The deposits most resembling unfixed, normal, adult, human dermis morphologically occurred when collagen was precipitated in the presence of CSA and keratosulfate (F1): the fibrils were rigid (no acute bends), long (few visible tapered ends) and 500 to 1000 A wide, being much narrower than the control (Fig. 16).

The greatest morphological effect was produced by heparin in a concentration of 0.005 per cent in the final mixture. Not only was macroscopic gelation prevented (Fig. 1), but the deposit was scanty and situated round the edges of the pools on the glass slides. Under the light microscope, star-shaped clusters of short wide tactoids dominated the picture in some areas, whereas others contained a fibrous network of long, thinner fibers with acute fibril bends and long tapered ends. Under the EM, both the tactoids and the compound fibers were of variable length and composed of fibrils 1000 to 1400 A in width. In addition, there was a characteristic background feltwork of fibrils 200 to 1000 A wide, the narrower examples bearing 220 A cross-striations. This was not seen in any other experiment. The 100-fold dilution (0.0005 per cent) of heparin failed to prevent macroscopic gelation, although the moderately heavy fibrous deposit contained a number of fibril bends and tapered ends.

It is well known that the stress produced during drying preparations for EM examination tends to flatten any tiny object, such as a virus particle (11). The collagen fibrils of unfixed, normal, adult, human dermis remain cylindrical after drying (as determined by the shadow) and appear rigid. Because of their indefinite length, this rigidity may be partly due to "pinning down" by other collagen fibrils farther along the deposit but beyond immediate view. It has been shown for reprecipitated collagen (1) that altering the experimental conditions (in particular raising the temperature to 37°C.) produces long, rigid, cylindrical fibrils similar to those found in fresh dermis. In the present study, a similar precipitate was formed in the presence of CSA or keratosulfate

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FIGURES 10 to 12

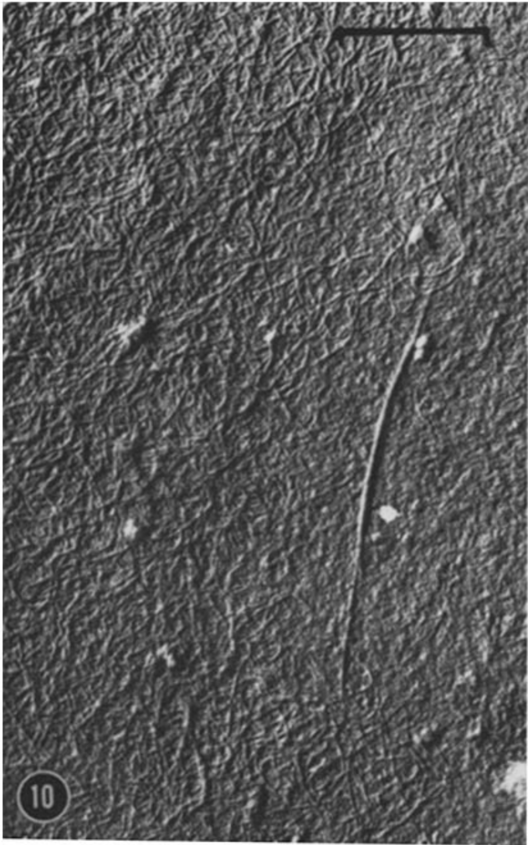
Replicated experiments employing DNA.

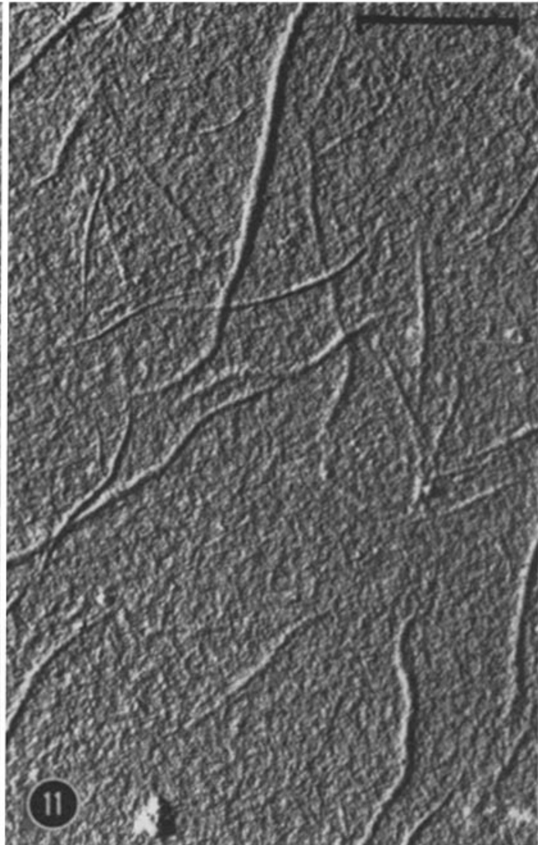
FIGURE 10

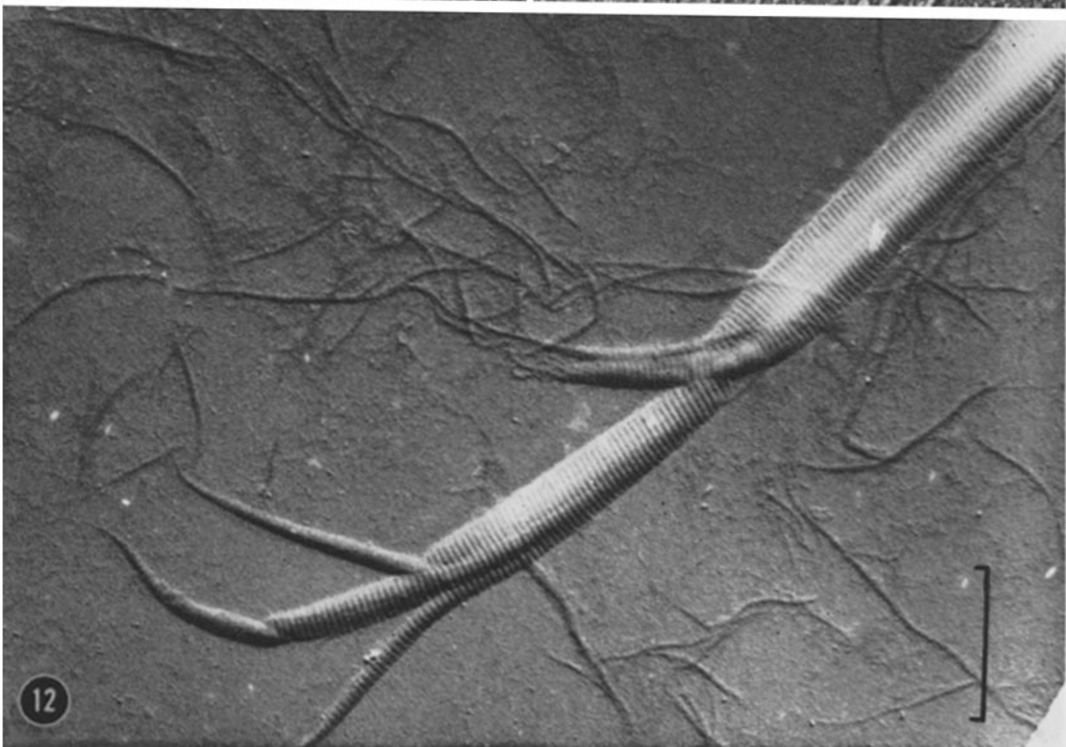
After 30 minutes using the whole dermal extract (Y_1), the deposit consisted of a heavy fibrous background with occasional non-striated filaments. After 1 hour scanty, striated tactoids appeared, but nothing was seen at the corresponding time intervals using the salt-extracted dermis (Y_2). × 20,000.

FIGURES 11 and 12

Illustrate the deposits after 3 hours using Dermis Y_2 and Y_1, respectively. The precipitate obtained from the salt-extracted material consisted only of the fibrous background and non-striated filaments (Fig. 11) whereas that obtained from whole dermal extract contained some wide, striated collagen fibrils and compound fibers in all stages of formation (Fig. 12). As the striated component increased the background material decreased in amount. × 20,000.







However, the control experiments, *i.e.*, precipitation by phosphate buffer alone, gave wider fibrils which exhibited a tendency to aggregate laterally in one plane (sheet formation; compound fibers) instead of the usual bundling that occurs with narrower fibrils. Part of this apparent increase in width, therefore, could be due to lack of rigidity allowing an elliptical cross-section to form on drying. This emphasizes the importance of investigating the incorporation of substances by collagen during its formation, their effects on the length, width, rigidity and stability of the resulting fibril, and their potential physiological application.

The collagen solution used in Part I of the present work had been prepared from salt-extracted dermis (Dermis X). The fact that the addition of one of the naturally occurring mucopolysaccharides (CSA) to the depleted Dermix X speeded up precipitation (3) and produced a deposit resembling unfixed, normal adult, human dermis supports the view that CSA aids normal fibril formation. The histograms of fibril width (Fig. 16) from the CSA experiments confirm the qualitative data recorded in Table I. Wood (3) found that the rate of precipitation during the first 2 hours, using CSA *b*, differed from that produced by CSA *a* and *c*. However, repeat EM experiments did not reveal any morphological difference in the deposit after 24 hours, all the purified samples of CSA giving a similar picture.

Nonstriated background filaments were present in variable quantities in most of the deposits, and were similar to those found occasionally in the electron micrographs of reprecipitated collagen by various authors. It has been suggested that these filaments aggregate to form the striated fibrils (7, 12). Although a constant feature of all the DNA experiments, they were quite different from the linear aggregates resembling branching "filaments" found in the air-dried sample of DNA alone. Many workers have published pictures of DNA and nucleoproteins using varying methods of preparation (13–19), all showing the tendency of this viscous solution of high molecular weight to aggregate in this manner.

As shown in Table II, both RNA and DNA influenced the rate of formation of collagen as well as the number and quality of the fibrils produced. This confirms Wood's investigations (3). Both the control and RNA experiments rapidly formed a gelatinous precipitate, which remained macroscopically unaltered throughout the 24 hours at room temperature. In contrast, the presence of DNA prevented gelation and only allowed the late formation of a flocculent precipitate at 9 hours with the whole dermal extract (Y_1), and a very slight opacity at 24 hours with the salt-extracted material (Y_2) (Fig. 6).

Under the EM, the presence of RNA decreased the proportion of collagen typical of unfixed, normal, adult human dermis, and, unlike the controls, the deposits remained in the same re-

FIGURES 13 to 15

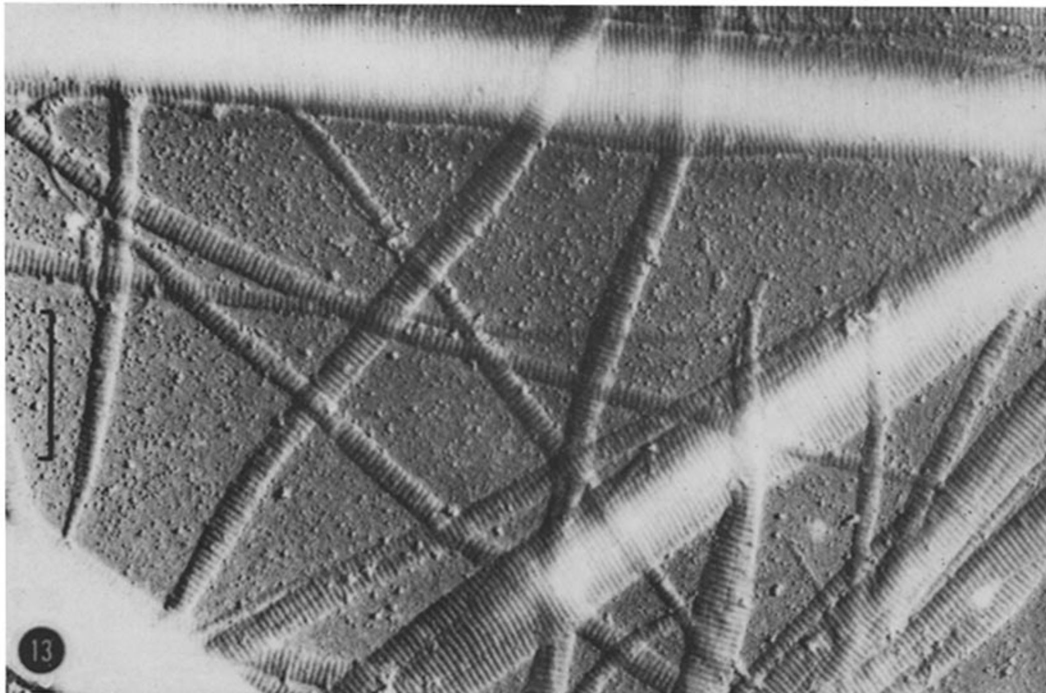
Replicated experiments employing DNA.

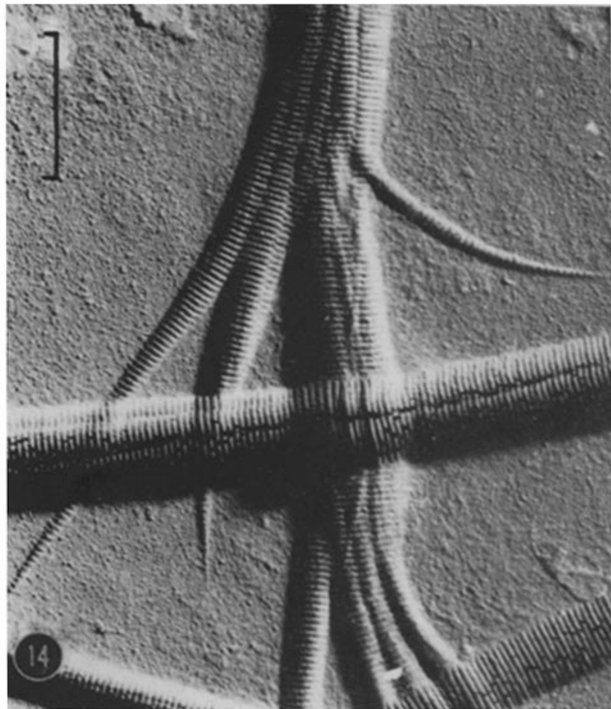
FIGURE 13

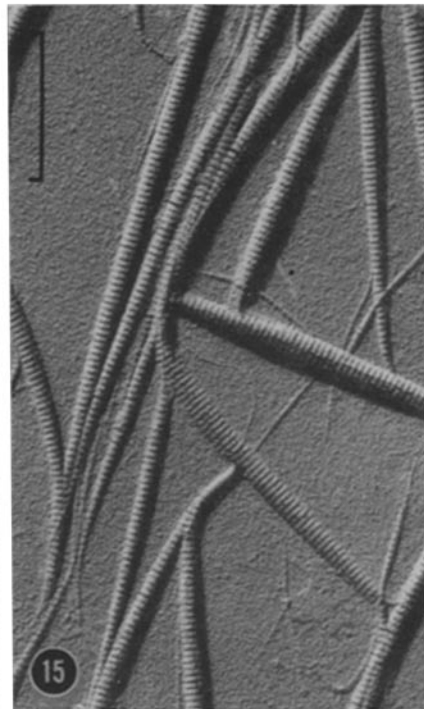
Illustrates some of the many long, wide, striated compound fibers and collagen fibrils seen after 9 hours utilizing the whole dermal extract (Y_1). The fibrous background and the filaments had disappeared. At the corresponding time interval the salt-extracted experiments still contained non-striated filaments, and the compound fibers were not so long or so wide as with Dermis Y_1. × 20,000.

FIGURES 14 and 15

The 24-hour deposit using the salt-extracted dermis (Y_2) contained some compound fibers and numerous short, slender tactoids 600 to 1,000 A in width. Both the fine fibrous background and scanty non-striated filaments were still present in some areas. The irregular longitudinal markings on the surface of the compound fibers in Fig. 14 is due to cracking of the metal replica owing to the thickness of the fibers. The denser 24-hour deposit employing Y_1 (not illustrated) was composed of large compound fibers and a background of narrow, striated fibrils. The thick compound fibers could not be adequately photographed, so only examples of moderate size are shown. × 20,000.







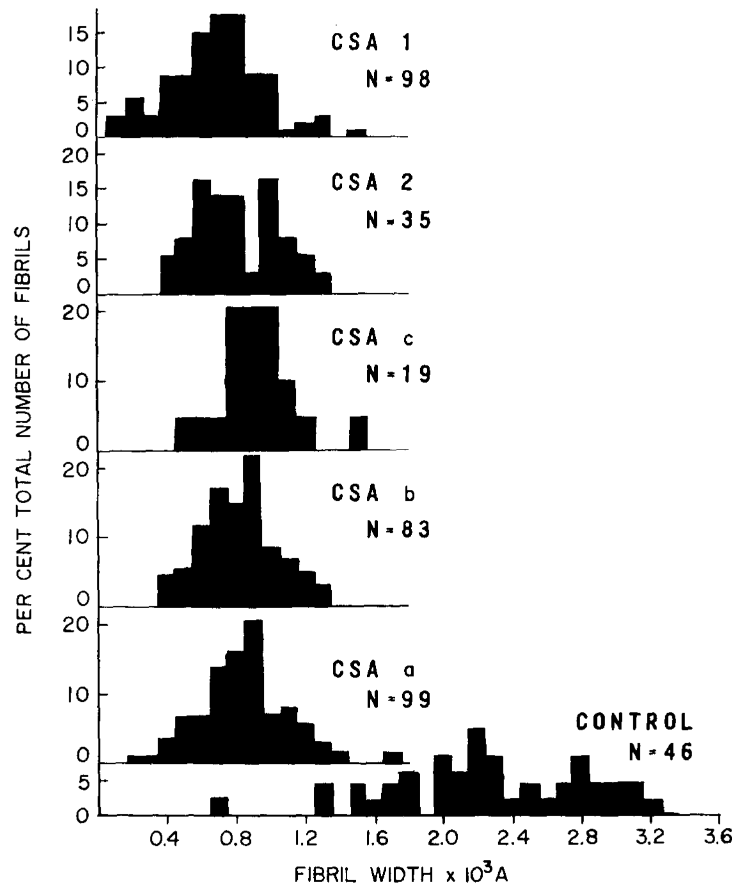


FIGURE 16

Measurements of fibril width (by G. C. Wood) on the EM photographic plates of the precipitates from the CSA and control experiments. CSA *1* and *2* obtained from Evans Medical Supplies and L. Light and Co., respectively. CSA *a* to *c* supplied by K. Meyer. N = number of fibrils measured in each experiment. There is no significant difference between the five CSA experiments, the most frequent fibril width being 600 to 1000 A. The control showed a much wider distribution of fibril width (1,300 to 3,200 A), the majority measuring 2,000 to 2,800 A.

tarded state throughout the 24 hours. These deposits were characterized by frequent fibril bends believed due to an abnormally pliable state of the fibrils as the suspension settled on the slide during drying.

DNA markedly interfered with the production of striated fibrils, this delay being much greater when the salt-extracted dermis was used. At 24 hours, there was a moderate deposit of structures similar to those found with the whole dermal extract (Y_1), but these were not so long, so large or so plentiful, and no striated background collagen fibrils were seen. Thus, DNA not only prevents the rapid formation of the normal long collagen fibrils found in the controls, but gradually forms abnormal, short, wide compound fibers and tactoids from a characteristic fibrous background not found in the control or RNA experiments. However, a small quantity of similar background material was seen throughout the RNA experiment employing Dermis Y_2, serving as an index of the retarded state of the deposits concerned.

The fact that the length of time the collagen solution has been stored before use affects the rate of collagen precipitation was briefly noted by Wood and Keech (1). The extract employed in Part II of the present study was prepared from freeze-dried, purified calf skin which had been stored for 9 months and from which it was becoming increasingly difficult to extract the soluble collagen, only 50 to 60 per cent being precipitated

under the experimental conditions used. On the other hand, the salt-extracted Dermis X used in Part I was freshly made, had not been stored, and was completely precipitated (1). This probably accounts for the less dense deposit of narrower fibrils in the control experiments of Part II as compared with the denser control deposit of wider fibrils exhibiting lateral aggregation obtained from Dermis X in Part I (Figs. 3 and 9). Thus, as already stated, collagen grading is only valid for comparative purposes within a particular set of experiments using the *same* collagen extract, as the morphological details in each grade may differ if another solution of collagen is employed. A repeat series of EM experiments employing an uncontaminated sample of stored collagen solution prepared from Dermis Y_2 revealed a less retarded rate of collagen formation in the presence of DNA, although the deposit consisted of structures similar to those recorded in Table II. This may be related to the spontaneous precipitation that occurs very slowly in extracts stored at refrigeration temperature (20). Although the stored extract used above had appeared clear and colorless to the unaided eye, it might have reached a physical state in which the DNA was less effective in delaying the reprecipitation of collagen. This serves to emphasize the importance of carefully repeating every aspect of experimental technique in order to obtain reproducible results.

The present investigation of the precipitates formed at serial time intervals employing collagen solutions prepared from whole dermis or salt-extracted dermis revealed that the former produced deposits containing more collagen resembling unfixed, normal, adult human dermis, more quickly in *each* set of experiments, as compared with the salt-extracted material. This suggests that the removal of the salt-soluble components of the dermis retards fiber formation.

Keech (20) demonstrated that collagen formed in the presence of DNA or heparin was rapidly rendered degenerate by mild mechanical stress in contrast to collagen reprecipitated in the presence of CSA or the fibrils of normal dermis. It would appear from this series of papers that very slight alterations in the local environment *in vitro* (such as temperature and ionic strength) and very slight alterations in the amounts of naturally occurring substances normally present in areas of collagen formation (mucopolysaccharides and nucleic acids) produce marked effects on the rate of precipitation, the stability and the morphology of the collagen fibrils formed.

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