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ULTRASTRUCTURAL STUDIES ON SOLUBILIZED AND RECONSTITUTED COLLAGENS.

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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

ULTRASTRUCTURAL STUDIES ON SOLUBILIZED AND RECONSTITUTED COLLAGENS

A DISSERTATION

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RALPH WILLIAM DAY

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ULTRASTRUCTURAL STUDIES ON SOLUBILIZED AND RECONSTITUTED COLLAGENS

APPROVED BY

DISSERTATION COMMITTEE

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ULTRASTRUCTURAL STUDIES ON SOLUBILIZED AND RECONSTITUTED COLLAGENS

CHAPTER I

INTRODUCTION

The discovery by deBroglie (1924) that a moving electron can be assigned a very short wave length and the demonstration by Busch (1926) that a suitably shaped magnetic or electrostatic field could be used as a true lens for an electron beam (Wischnitzer, 1962) initiated a line of investigation that led to the almost simultaneous construction of the electron microscope by Ruska and Knoll and by Ruedenberg in 1931. Marton (1934) was the first to apply electron microscopic techniques to biological objects (Wyckoff, 1949) but a working resolution of about 50 A was not achieved until the early nineteen-forties (Schmitt, 1944-45). Use of the electron microscope to study connective tissue was attempted at this time and the cross-striated banding pattern in collagen fibrils was described by Schmitt, Hall and Jakus (1942).

Zacharides (1900) noted that rat tail tendon could be dissolved in dilute acetic acid and Nageotte (1927) precipitated fibers that had the properties of native collagen from such solutions. Examination of connective tissue extracts

with electron microscopic techniques soon led to the discovery of fibrous-long-spacing (FLS), (Highberger, Gross and Schmitt, 1950) and segment-long-spacing (SLS), (Schmitt, Gross and Highberger, 1953) types of collagen, neither of which occur in nature (Cox, Grant and Horne, 1967).

A major problem in collagen research has been concerned with the morphology of the collagen molecule and its aggregation into fibrils. Schmitt (1944-45) suggested two possiblities for the formation of fibers; (1) by lateral and longitudinal aggregation of extremely thin preformed fibrous units, possibly the highly elongated native protein molecules themselves, or (2) by columnar aggregation of relatively symmetrical soluble globular molecules. The generally accepted theory at present assumes the existence of a fundamental collagen particle that occurs in the form of discrete monomeric units (Schmitt, et al., 1955; Hodge and Schmitt, 1961) named "tropocollagen" by Gross, Highberger and Schmitt (1954). From its physicochemical behavior, it is assumed to be an elongated, stiff, rod-shaped macromolecule approximately 14 A in diameter and 3000 A long with a molecular weight of 300,000 (Gross, et al., 1954; Jackson, 1958; Boedtker and Doty, 1954, 1955, 1956; Hodge and Schmitt, 1960). No such particle has ever been demonstrated in intact connective tissue or collagen fibrils. Electron microscopic studies of shadowed preparations of "tropocollagen" (Hall, 1956; Hall and Doty, 1958; Rice, 1961) have yielded pictures of long, filamentous units which have been interpreted to be fundamental building blocks of collagen. Schmitt, Gross and Highberger (1955) believed these elongated particles to be the "kinetic unit" of collagen. Therefore, the

length of an SLS segment was thought to be the length of a tropocollagen molecule, the band pattern of SLS being a "molecular fingerprint" of the basic monomeric unit of collagen with many tropocollagen molecules aggregated side-by-side and with levels of order in register (Hodge and Schmitt, 1960; Schmitt and Hodge, 1960; Hodge, et al., 1960; Hodge and Petruska, 1962, 1963; Petruska and Hodge, 1964; Cox, et al., 1967).

Examination of shadowed preparations published by Hall (1956), Hall and Doty (1958) or Rice (1961) reveals a "beaded" pattern along the "filaments" that were thought to be tropocollagen plus globular particles of the same size as the "beaded" elements scattered among the filamentous structures. These globular units were apparently ignored by the authors and subsequent investigators. Also, the histogram of about 105 measurements of tropocollagen lengths in the original publication by Hall (1956) revealed a wide distribution ranging from about 500 A to 4000 A without any pronounced peak but an average of about 2200 A. It seems reasonable to expect length measurements of the collagen molecule to fall within a relatively narrow range and, if SLS is to be a "fingerprint" of this molecule, the lengths of tropocollagen and SLS should be in close agreement. Using negative staining techniques, Olsen (1967) found a range of 2800 A to 3400 A with a mean of 3130 A, and Gross (1956) reported lengths averaging 2400 A in postively-stained material. Therefore, a range of SLS lengths varying by at least 1000 A has been reported.

Several alternatives to the tropocollagen theory have been proposed. Studies of native type, formaldehyde-treated and chromium-tanned collagen fibrils

by shadow casting, phosphotungstic acid positive staining and sodium tungstate negative staining led Borasky and Shimony (1965) to postulate a fundamental structural element in collagen that is 650 A long. X-ray diffraction studies by Cowan, North and Randall (1955) yielded data which they interpreted as requiring a "spherical or ellipsoidal diffractor of length 210-240 A." The "procollagen" molecule extracted from connective tissues by citrate buffers was deduced by Bresler, using physicochemical methods, to be a cylindrical macromolecule with a diameter of about 17 A, length 380 A and a weight of about 70,000. There was thought to be a polypeptide chain approximately 2400 A long coiled within this "cylinder" (Highberger, Gross and Schmitt, 1951). The extraction of calf skins with phosphate buffer and the subsequent salting-out of the collagen with saturated ammonium sulfate solution yielded small globular units (Kahn, Carroll and Witnauer, 1962). This precipitate could be resolubilized and again salted-out until a type of banded fibril was produced. Richter, et al. (1969) have demonstrated the presence of a globular unit in model dog scar tissue, tendon, etc., having a pear-shaped configuration with an over-all length of about 270 A, a width at the bulbous portion of 108 to 116 A and a width in the thin part averaging 57 A. Davidovits (1966) proposed a "macromolecular micelle" model for collagen consisting of a macromolecular chain wound into a spiral which consists of straight lengths of chains and curves, the general form being a planar ovoid disk which is able to swell in solution. Furthermore, he states, "The rod-like structure of tropocollagen is considered to be a special denatured form of collagen uniquely obtained under certain conditions of temperature."

Gross described time studies of FLS formation in which the early stages are characterized by "clouds of dense spheroids." These disappeared with increasing time of precipitation and were replaced by typical FLS fibrils. SLS also tended to form "densely staining spheroidal centers about 500 A in diameter" (Schmitt, et al., 1955; Gross, 1956).

Most micrographs of collagen fibrils show a "globular" structure occurring randomly among the fibrils. These are generally ignored in the descriptions of collagen morphology. Cox, Grant and Horne (1967) state that, "the part played by the long linear protofibril in the formation of the native-type collagen fibril (Hodge and Schmitt, 1960; Hodge and Petruska, 1963; Hodge, et al., 1960; Olsen, 1963b) has been too readily accepted." Human dermal collagen fibers fragmented in a Waring blender show globular units near the frayed ends of fibrils where some separation has occurred between subunits; similar particles are distributed randomly among the fibrils in the micrographs done by Grassman and his coworkers (1957).

Extensive preliminary studies in our laboratory on extracts of model scar tissues from the dog, rat tail tendon, dog tendon and skin and carp swim bladder tunic have consistently revealed a globular-fine structure in reconstituted collagen preparations. These findings, the often ignored data in the literature and the prevalent assumption that SLS is a "molecular fingerprint" of the collagen molecule led to the choice of SLS preparations as a model for investigating collagen ultrastructure. An attempt has been made to evaluate the ultrastructural development of the SLS type of collagen fibril by conducting a time study of SLS formation.

CHAPTER II

MATERIALS AND METHODS

Materials

The biological material consisted of collagen extracted from the tunica externa of the carp (Cyprinus carpio) swim bladder. The tunics were fragmented in a Sorvall Omni-Mixer (Ivan Sorvall, Inc., Norwalk, Connecticut). Low-speed centrifugations were performed in an International bucket-type centrifuge having a radius of 24 cms in a cold room of 8° C or less. High speed centrifugations were carried out in a Spinco preparative ultracentrifuge at 5°C. Visking dialysis tubing 7.5 cm flat width, wall thickness 0.0016 inch, with an average pore diameter of 24 A was used (Union Carbide).

Sodium acetate was prepared as a 0.5 molar solution in distilled water. The latter was purchased from Cutter Laboratories, Berkeley, California, as sterile water for injection, USP. Citrate buffer, pH 4.3 contained 0.1 M citric acid and 0.1 M sodium citrate.

Specimen screens were 300 mesh copper grids purchased from Ernest F. Fullam, Inc., P. O. Box 444, Schenectady, New York. Supporting films were made from polyvinyl formal plastic, "Formvar," (Shawinigan Resins Corporation, Springfield, Massachusetts). Embedding plastics were a mixture of

ethyl methacrylate monomer (20 parts) and butyl methacrylate monomer (80 parts). The polymerizing agent or catalyst was 3% benzoyl peroxide. Embedding capsules were obtained from BEEM (Better Equipment for Electron Microscopy, Inc., P.O. Box 132, Jerome Avenue Station, Bronx, New York). Ultrathin sections were cut on the MT-1 Porter-Blum Microtome using conventionally prepared glass knives.

Positive staining of sectioned and precipitated material utilized 1% w/v aqueous phosphotungstic acid (PTA) solutions, 0.5% w/v aqueous uranyl acetate (UA) solutions, or combinations of these two. Negative staining methods utilized a 2% w/v aqueous PTA adjusted to pH 7.0 with 1 N KOH or exposure to the vapors of 1% w/v aqueous osmium tetroxide (OsO_4).

Electron micrographs were prepared using the RCA-EMU4 electron microscope. Photographic enlargements were made with the Simmon Omega variable condenser model D2 enlarger on Kodak Kodabromide single weight photographic paper. Instrument magnifications were calculated from micrographs of carbon grating replicas of 28,800 and 54,800 lines per inch.

Methods

Preparation of Supporting Films

Formvar supporting films were prepared by freshly diluting a 1% w/v stock solution of Formvar in 1,2-dichloroethane to make a 0.25% solution. Clean microscope slides were dipped in the Formvar solution contained in a 100 ml graduated cylinder, and then suspended above it for 45 seconds before

removal. The edges of the air dried slides were then scored with a razor blade and the plastic film floated off the slide onto a clean distilled water surface. Specimen screens were placed on the film and a clean plastic centrifuge tube rolled across the water surface to pick up the film, trapping the grids beneath it. These were dried in air over Drierite in a vacuum jar prior to use. The specimen screens were then grasped by one edge with watchmaker's forceps and lifted from the tube with their plastic coating intact.

Ichthyocol

Ichthyocol, acid-extracted carp (Cyprinus carpio) swim bladder tunics, was prepared by the method of Gallop (1955) modified slightly as follows. All procedures were carried out on solutions kept at 4-8°C. One-hundred grams of swim bladder tunics were placed in 400 ml of 0.5 M sodium acetate and fragmented in a Sorval Omni-Mixer for three minutes. The resulting paste was refrigerated for 24 hours and then centrifuged in a cold room at 2000 to 2500 revolutions per minute for one hour. The centrifugate was resuspended in 400 ml of cold distilled water and refrigerated for 24 hours followed by centrifugation at 2000 RPM for one hour.

The final centrifugate was then immersed in 400 ml of pH 4.3 citrate buffer and refrigerated for 24 to 48 hours. The citrate extract was centrifuged at 2000 RPM for 2 hours and the supernatant filtered through Whatman No. 1 filter paper, using 15 mm Hg negative pressure. The filtrate was centrifuged at 50,000 Xg for 2 hours at 5°C. The resulting supernatant was placed in dialysis bags and suspended in 80 liters of cold 0.2 M disodium phosphate for

48 hours. The dialysis fluid was kept in motion by a magnetic stirrer. The dialyzed material was washed in three changes of distilled water, 200 ml each, centrifuging at 2000 RPM for 30 minutes. The final ichthyocol preparation was stored in distilled water at 4-5°C and referred to as the stock ichthyocol.

Segment-Long-Spacing Collagen Fibrils

Segment-long-spacing collagen fibrils (SLS) were prepared from the stock ichthyocol by centrifuging 8 ml of suspension at 2000 RPM and discarding the supernatant solution. Two milliliters of cold 0.1% acetic acid solution was added to the ichthyocol pellet and the collagen solubilized. One milliliter of 0.22% ATP in 0.1% acetic acid was added to give a final ATP concentration of 0.073%. This treatment induced the precipitation of the ichthyocol as SLS segments. It should be noted that prolonged exposure of copper specimen screens to the acetic acid used in these preparations caused disintegration of the screens. Acetic acid has been used frequently as a volatile solvent in attempts to visualize small molecules (Beer, 1961; Beer and Zobel, 1961) but this effect has not been reported previously. Acetic acid solutions were found to change the Formvar films with prolonged exposure also. The specimens in this study were air-dried in a few seconds and the Formvar films were coated with carbon prior to use. Therefore, no artifactual changes attributable to the acetic acid could be demonstrated.

Sectioned SLS

SLS was prepared for examination by embedding and sectioning

techniques. The SLS (ichthyocol-ATP) solution was centrifuged at 2000 RPM in the cold after 24 hours of polymerization and the pellet was dehydrated in absolute alcohol overnight. After a second centrifugation, the pellet was suspended in embedding methacrylate-ethanol (50:50) mixture for 2 hours. The material was subsequently centrifuged and the pellet was embedded in a methacrylate mixture containing 20 parts ethyl methacrylate to 80 parts butyl methacrylate with 3% benzoyl peroxide added as a catalyst. Polymerization in the embedding capsules was effected by exposure to ultraviolet irradiation.

Time-Study by the Loop Method

A second method of specimen preparation involved placing drops of the ichthyocol-ATP mixture on carbon-coated Formvar-coated specimen screens with a 3 mm diameter wire loop starting 5 minutes after adding the ATP and continuing for 10 minutes. A total of 111 time-sequenced grids was prepared. These collectively represent samples of the reconstituting system at an average time interval of 5.45 seconds. The objective was to obtain data on the mode of aggregation of the ichthyocol molecules into SLS forms of collagen.

Time-Study by the Spray Method

A third method of specimen preparation utilized a DeVilbiss number 15 nebulizer activated by an EFFA aerosol duster to spray the ichthyocol-ATP solution at less than 8^{0}C onto the carbon-coated Formvar screens. This was done as a time-study starting 5 seconds after adding ATP to the ichthyocol solution (zero time) and then spraying ten specimen screens each at 5 second intervals to

one minute, then at 70, 80, 90, 100, 110, 120, 140, 160, 180, 220, 260, 340, 420 and 500 seconds from zero time. The specimens were air-dried following the spraying.

Positive Staining

Specimens were prepared for examination in positive contrast by simply floating the specimen screen with its section or precipitated materials on the PTA or UA staining solutions for varying time periods to obtain an empirically adequate staining effect. The specimens were then washed by floating them on distilled water for one hour. The staining solution was either placed in the depression of a spot plate or placed as a drop on a paraffin surface.

Negative Staining

Two methods were developed to achieve a negative contrast effect. The first used a 2% w/v aqueous PTA solution adjusted to pH 7.0; this was applied to the specimen on a copper grid by using a 3 mm diameter wire loop to deposit a thin film of the stain. Thirty seconds later the edge of the grid was touched with filter paper to remove excess fluid and the preparation was air-dried.

The second method was a modification of that reported by Barland and Rojkind (1966) utilizing osmium tetroxide. A clean glass microscope slide was prepared by sticking a one-fourth inch wide strip of double-surfaced masking tape across one end. The edge of a grid bearing the specimen was then pressed to the edge of the tape. The slide with its attached screens was dropped, specimen end up, into a Coplin jar containing ten milliliters of 1% w/v aqueous OsO_4 at

room temperature. The specimen was therefore exposed only to the vapors of the OsO_4. Staining time varies with the specimen and had to be determined empirically.

CHAPTER III

OBSERVATIONS

Representative micrographs from the time-study of SLS formation, in which specimen screens were sprayed at stated time intervals after adding adenosine triphosphate (ATP) to the stock ichthyocol solution, are demonstrated in Plates I through XI in the Appendix (all Plates and Figures referred to in this dissertation will be found in the appendix which begins on page 54). The figures are organized in an increasing time sequence starting with five seconds and ending at five-hundred seconds (8 1/3 minutes); Plates I through XI, Figs. 1 through 41. Micrographs from the time-study by the loop method follow and include a time range from 5 1/3 minutes to 75 minutes; Plates XI through XV, Figs. 42 through 55. Examination of these figures in sequence reveals that the details of the SLS vary significantly during fibrillogenesis.

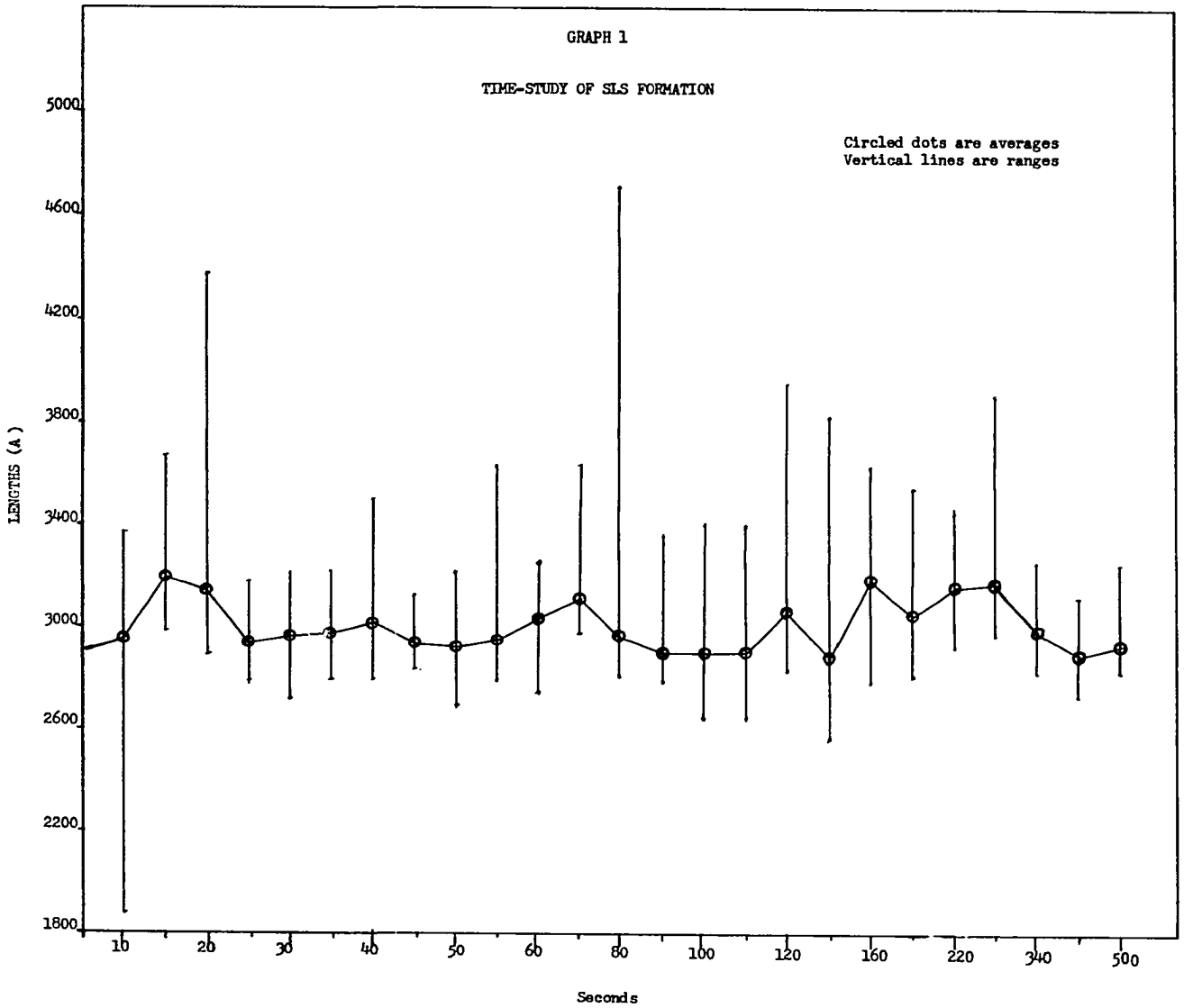
Length and Width Data

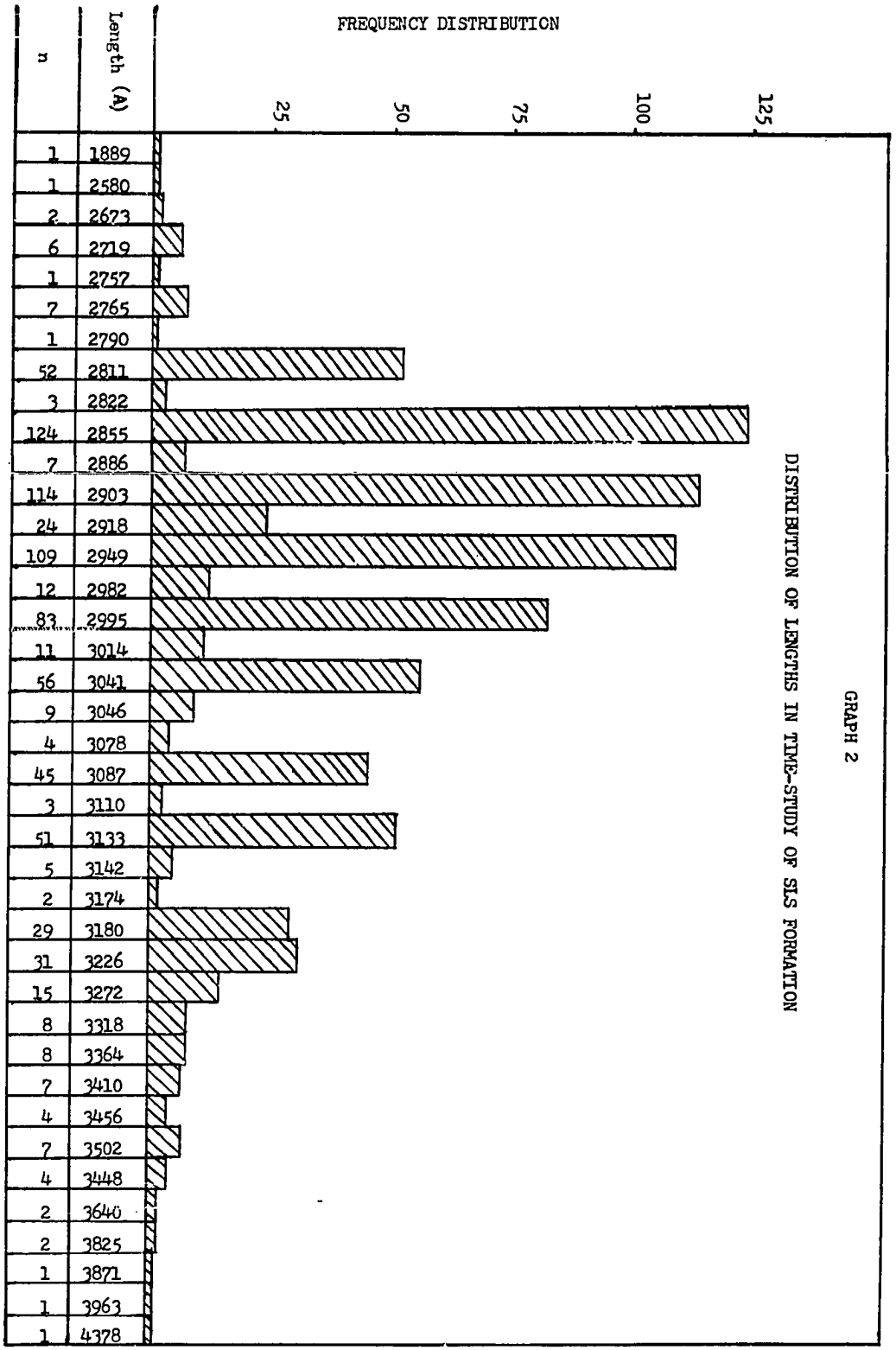
The lengths and widths of the immature SLS fibrils formed at each time period were measured; the results are summarized in Tables 1 and 2 and in Graphs 1, 2, 3, and 4. This type of data may be misleading in some respects since the immature SLS segments are not always discrete, uniform structures, as an examination of the early figures will reveal. Therefore, the choice of

TABLE 1

TIME-STUDY OF SLS FORMATION

		Lengths (A)			
Seconds	Number of SLS Measured	Shortest	Average	Longest	Longest minus Shortest
5	1		2903		
10	6	1889	2958	3410	1521
15	14	2995	3207	3686	691
20	23	2903	3152	4378	1475
25	50	2811	2940	3180	369
30	44	2719	2977	3226	507
35	23	2811	2986	3226	415
40	39	2811	3032	3502	691
45	16	2857	2949	3133	276
50	30	2719	2931	3226	507
55	25	2811	2958	3087	276
60	26	2765	2885	3041	276
70	30	2995	3133	3825	830
80	61	2811	2968	3502	691
90	56	2811	2912	3180	369
100	40	2673	2912	3180	507
110	35	2673	2921	3410	737
120	43	2857	3087	3963	1106
140	74	2580	2903	3825	1245
160	21	2811	3216	3640	829
180	22	2857	3078	3548	691
220	28	2949	3180	3456	507
260	42	2995	3189	3548	553
340	39	2854	3014	3174	320
420	29	2757	2911	3142	385
500	36	2854	2950	3078	224
n	853				
Average			3010		
range		1889		4378	


GRAPH 1
TIME-STUDY OF SLS FORMATION
Circled dots are averages
Vertical lines are ranges
LENGTHS (A)
5000
4600
4200
3800
3400
3000
2600
2200
1800
10
20
30
40
50
60
80
100
120
160
220
340
500
Seconds



GRAPH 2

DISTRIBUTION OF LENGTHS IN TIME-STUDY OF SLS FORMATION

where to measure the segments was often arbitrary, although these decisions were made on all micrographs before any measurements were taken. Plate II, Figs. 2, 3 and 4 illustrate this difficulty. In some cases, for example, the length could be measured at two different points. In Plate II, Fig. 4 there is significant variation in the length of the segment along the border labeled A compared with the side labeled B. Also, much of the filamentous material in the so-called extra-fibrillar area appears to be continuous with the fibril.

Nevertheless, an attempt was made to evaluate the lengths of the SLS in the time-study conducted by the spray method; the shortest, average and longest lengths at each time period and the number of fibrils measured are listed in Table 1. A pictorial representation of these results is presented in Graph 1. The lengths measured in the total sample of 853 fibrils ranged from 1889 A to 4378 A, a spread of 2489 A, while the over-all average was 3010 A. A large majority of these values (92.5%) clustered in the range from 2811 to 3272 A, as demonstrated in Graph 2. The greatest range of lengths at any time interval occurred at 80 seconds with a difference of 1889 A and the least difference was 276 A at 45 seconds (Graph 1).

It has been suggested that the alternating profile of the curves in Graphs 2 and 4 represents bias in the data. The measurements were estimated to the nearest 0.5 mm or approximately 46 A at the magnification used in this study. This problem was discussed with Dr. Arthur Nunnery, biostatistician at the University of Oklahoma Medical Center; no statistical methods were found that might test the validity of the data. Rather than mask the possible bias by

combining adjacent values on the graph, the actual lengths were recorded. Since only the range and mean values of the distribution are emphasized in the discussion, this possible bias should have little effect on the interpretations.

Great variation in SLS width occurs along the length of a given segment (Plate I, Fig. 1; Plate III, Fig. 6; Plate IV, Figs. 8, 10 and 11; Plate V, Figs. 12 and 13; Plate VII, Figs. 22, 23, and 25; Plate VIII, Figs. 27, 28, 30, 31, 32, 33, and 34; Plate X, Fig. 37; Plate XI, Fig. 40). The least and the greatest diameters were measured on each SLS segment. These data are summarized in Table 2 and Graphs 3 and 4. An examination of many micrographs revealed only one fibril in the 5 second time period (Plate I, Fig. 1). It should be pointed out here that although the specimen screens were sprayed with the ichthyocol-ATP mixture at a stated time after the sample preparation, a period of time occurred during the air-drying process in which SLS fibrillogenesis could have continued. Therefore, the exact age of any SLS segment cannot be determined exactly. However, since the drying time lasted only a few seconds and the time-study encompassed several minutes, the general sequence of fibrillogenesis and the changes that occur can still be demonstrated reliably.

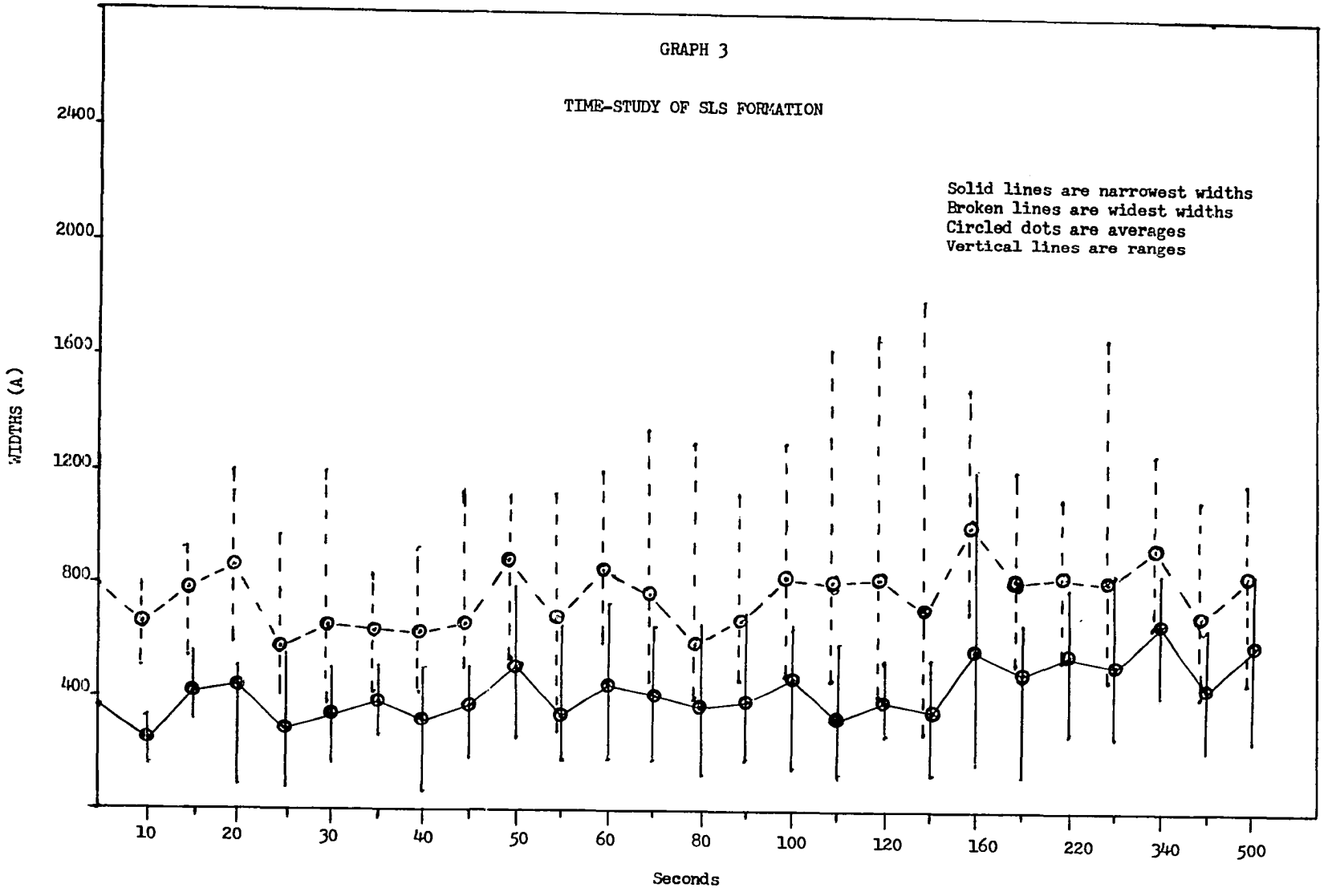
The sample of 1685 measurements on 853 SLS segments showed widths ranging from 92 A to 1797 A (Table 2, Graphs 3 and 4). Evaluation of the "narrowest point" data reveals a range of 92 A to 1198 A with an over-all average of 420 A. The "widest point" data show a spread of 276 A to 1797 A with an over-all average of 760 A. The over-all spread of widths, therefore, was 1705 A with a spread of 1106 A (1198 A to 92 A) for the "narrowest point"

TABLE 2

TIME-STUDY OF SLS FORMATION

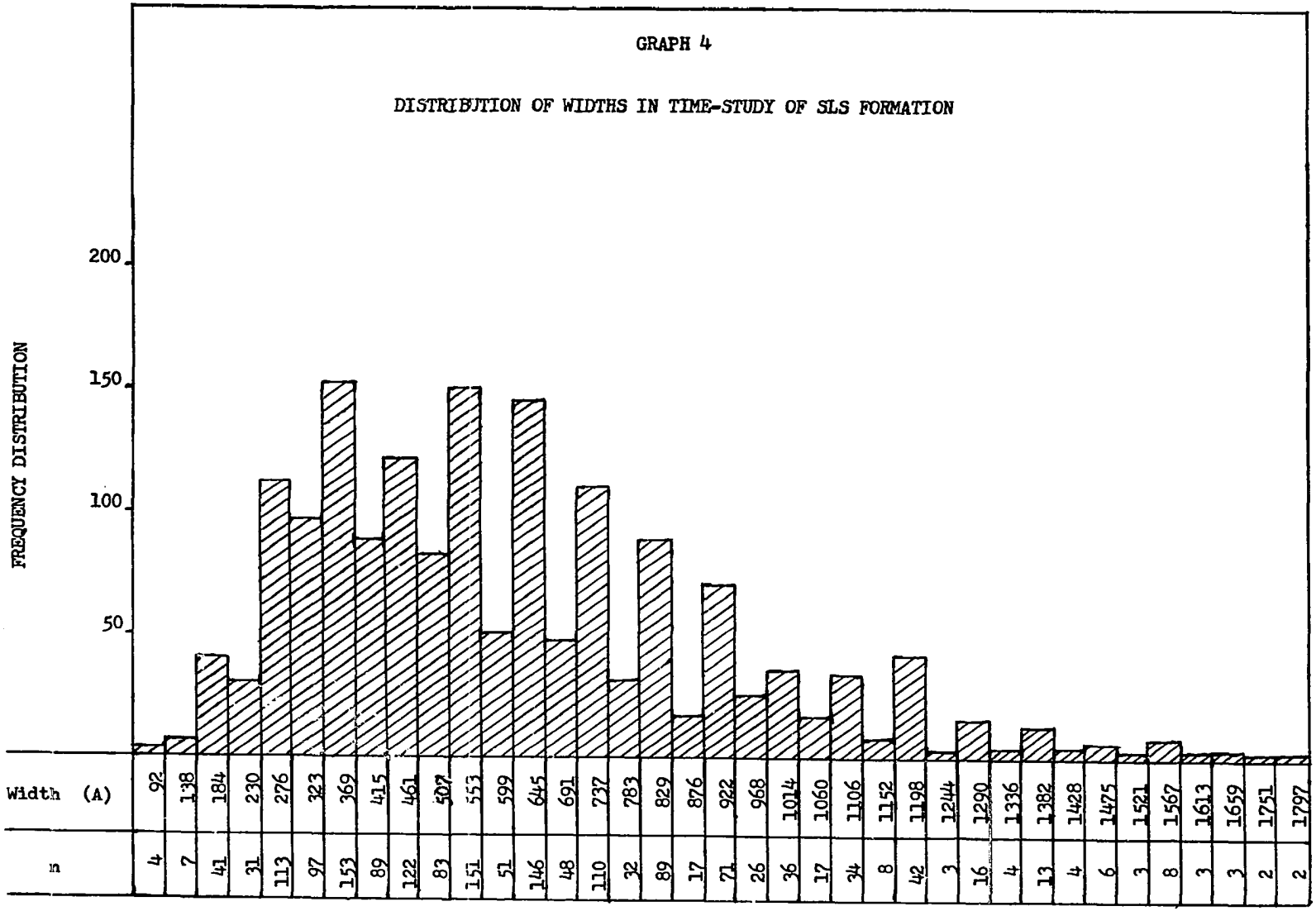
		Widths (A)							
		Narrowest Point				Widest Point			
Seconds	Number of SLS Measured	L	Ave.	G	G-L	L	Ave.	G	G-L
5	1		369				783		
10	6	184	258	323	139	507	664	783	276
15	14	184	276	369	185	553	737	1106	553
20	23	92	442	507	415	599	876	1198	599
25	50	92	286	553	461	415	581	968	553
30	44	184	341	507	323	369	654	1198	829
35	23	276	396	507	231	415	636	829	414
40	39	92	323	507	415	415	627	922	507
45	16	184	378	507	323	507	654	1106	599
50	30	276	525	783	507	461	894	1106	645
55	25	184	341	645	461	276	682	1106	424
60	26	184	452	737	553	599	857	1198	599
70	30	184	415	645	461	461	774	1336	875
80	61	138	369	645	507	323	590	1290	967
90	56	184	387	691	507	461	673	1106	645
100	40	92	470	645	553	461	829	1290	829
110	35	138	332	599	461	461	811	1613	1152
120	43	276	396	533	257	415	820	1659	1244
140	74	138	350	533	395	276	710	1797	1521
160	21	184	581	1198	1014	691	1014	1475	784
180	22	138	488	645	507	533	802	1198	665
220	28	276	562	783	507	533	839	1106	573
260	42	276	507	829	553	461	802	1659	1198
340	39	417	648	834	417	641	936	1250	609
420	29	224	436	641	417	417	693	1090	673
500	36	257	584	834	577	449	827	1154	705
n	853								
average			420				760		
range		92		1198		276		1797	

L - least width measured
G - greatest width measured
Ave. - average of all widths
G-L - difference between widest and narrowest SLS


GRAPH 3
TIME-STUDY OF SLS FORMATION
Solid lines are narrowest widths
Broken lines are widest widths
Circled dots are averages
Vertical lines are ranges
WIDTHS (A)
2400
2000
1600
1200
800
400
10
20
30
40
50
60
80
100
120
160
220
340
500
Seconds

GRAPH 4

DISTRIBUTION OF WIDTHS IN TIME-STUDY OF SLS FORMATION



Width (A)	92	138	184	230	276	323	369	415	461	507	553	599	645	691	737	783	829	876	922	968	1014	1060	1106	1152	1198	1244	1290	1336	1382	1428	1475	1521	1567	1613	1659	1751	1797
n	4	7	41	31	113	97	153	89	122	83	151	51	146	48	110	32	89	17	71	26	36	17	34	8	42	3	16	4	13	4	6	3	8	3	3	2	2

data and 1521 A (1797 A to 276 A) for the "widest point" data. As shown in Graph 3, there is considerable overlap between width values for the narrowest and widest points of segments at any given time period. If the analysis is restricted to the stated time periods rather than lumping all of the data for the time-study, then the least difference in widths occurred at 10 seconds with a spread of 139 A for the "narrowest point" data and a spread of 276 A for the "widest point" measurements. The greatest difference for the "widest point" values is 1521 A in the 140 second material. It is interesting to note that the narrowest width recorded was 92 A (Table 2), a value close to the width of the bulbous part of the pear-shaped structure described by Richter and Schilling (1969, 1970). Graph 4 reveals that whole-number multiples of this 92 A value recurred in 68.8% of the 1685 measurements. A decision as to whether this correlation is an artifact of the methodology or a result of the inherent morphology of the collagen molecule must await further analysis, preferably statistical in nature and using micrographs taken at greater magnification than those in this study.

There is significant variation in the length of the developing SLS segments, not only at different time periods but also among SLS in a given micrograph (Plate II, Fig. 5; Plate XIV, Fig. 52). This is demonstrated most clearly in Plate XIV, Fig. 52 in which segments A, B and C are each 1824 A in length at the middle of each fibril, whereas segment D is 2292 A long. This represents a length difference of about twenty per cent between segment D and any one of the other three SLS. Even more unexpected was the finding that the developing SLS fibrils varied in length across their own width (Plate II, Figs. 4 and 5;

Plate IV, Fig. 11; Plate VII, Fig. 24; Plate VIII, Figs. 28 and 30; Plate IX, Fig. 36). Plate II, Fig. 4 demonstrates a significant variation in the length of a segment along the border labeled A compared with that labeled B. A similar configuration is noted in Plate II, Fig. 5 in a shorter segment labeled A. The fibril labeled B in Plate VII, Fig. 24 at 100 seconds is a more mature, uniform segment, yet it is much longer on one side than on the other.

Changes in SLS Formation

A survey of several grids and dozens of grid areas yielded only one discrete segment at the 5 second time period (Plate I, Fig. 1). As noted earlier, the time required for the air-drying process could have increased the time available for fibrillogenesis by a few seconds. There are a few fibrillar structures in this micrograph but they are very short, irregular in shape and do not conform to the classical description of an SLS fibril; they may be early stages in the formation of SLS segments. The overwhelming characteristic of the 5 second preparation, however, is the abundance of globular structures of various sizes and shapes and the bead-like ultrastructural detail that is common to all the globular and filamentous elements. The magnification used in this study is too low to allow accurate measurements on the bead-like units but they are estimated to be in the range of 45 to 65 A in cross section. They are present in both SLS forms and globular elements in all micrographs taken during this study.

The larger globular units shown in Plate I, Fig. 1 are consistently seen throughout the time-study and in "mature" SLS preparations (note Plate IX, Fig. 41, SLS at 8 1/3 minutes and Plate XIV, Fig. 52, SLS at 75 minutes).

The globules are discernable in negatively and positively stained and in shadow-cast preparations (note arrow in Plate XIII, Fig. 48). Plate II, Fig. 2, SLS at 10 seconds, shows a predominance of globular units. The few fibrillar elements present appear to be aggregates of these globular structures. By 15 seconds, Plate II, Figs. 3, 4 and 5, the fibrillar elements have become more uniform and "SLS-like" in profile with rounded projections occurring along their borders (see A in Plate II, Fig. 3; A and B in Plate II, Fig. 5) with globular structures present in the extra-fibrillar material and in the splayed ends of the fibrils (structures labeled A in Plate III, Figs. 6 and 7).

As one surveys the sequence of developmental stages, the SLS segments become more uniform in profile in older material (compare Plate VI, Fig. 15 with Plate XI, Fig. 41) and the number of segments appears to increase relative to the non-fibrillar material (compare Plate III, Fig. 7 with Plate X, Fig. 37). The SLS fibrils in Plate XIV, Fig. 52 represent "mature" SLS similar to those classicallydescribed in the literature (Schmitt, Gross and Highberger, 1953). If the reconstituting SLS suspension is kept at refrigerator temperatures for several months, however, the very wide SLS segments shown in Plate XV are found. Many of the SLS at 3 1/2 months will appear to be two segments joined end-to-end (Plate XV, Fig. 53). Most segments, however, will be single SLS forms of extreme width (Plate XV, Figs. 54 and 55).

The overt globularity of the segments is obscured early in fibrillogenesis. The segment at A in Plate IV, Fig. 11, a 45-second preparation,

demonstrates a globular substructure throughout the whole fibril. At 500 seconds, the extra-fibrillar areas contain numerous isolated globular structures. The fibrils are large and relatively uniform in their profiles (Plate XI, Fig. 41) with many globular or rounded projections associated with their lateral margins (structure A in Plate XI, Fig. 41). The small bead-like feature mentioned earlier is evident within the ultrastructure of the fibril also.

Rounded projections of a size similar to the extrafibrillar globules occur along the margins of SLS segments from the very early to the more mature stages of fibrillogenesis (Plate II, Figs. 2, 3, 4 and 5; Plate III, Figs. 6 and 7; Plate IV, Figs. 8, 9, 10 and 11; Plate V, Figs. 12, 13 and 14; Plate VI, Figs. 15, 16 and 17; Plate VII, Figs. 21, 22, 23, 24, 25 and 26; Plate VIII, Figs. 27, 28, 30, 31, 32 and 33; Plate IX, Figs. 35 and 36; Plate X, Figs. 37 and 38; Plate XI, Figs. 39, 40 and 41; Plate XII, Fig. 43; and Plate XIV, Fig. 52). This is especially noticeable in the structure labeled B in Plate V, Fig. 12. In the young segments the ends are sometimes rounded rather than straight or slightly curved as is typical of the more mature fibrils (compare the rounded ends of the segments in Plate II, Fig. 3; Plate III, Fig. 6; Plate IV, Figs. 8, 10 and 11; Plate V, Fig. 13; Plate VI, Figs. 15 and 16; Plate VII, Figs. 23 and 24; Plate VIII, Figs. 27, 28, 30 and 31; Plate IX, Fig. 36; and Plate X, Fig. 37) with the ends of the SLS in Plate III, Fig. 7; Plate VII, Fig. 21; Plate VIII, Fig. 34; Plate XI, Figs. 39, 40, 41 and 42; Plate XII, Figs. 43, 44, and 46; and Plate XIV, Fig. 52). This rounding of the ends of SLS fibrils nearly always occurs at only one end of a segment.

Occasionally in these SLS preparations, globular structures will be

found to be either aggregated in rows that suggest an early stage of SLS formation (see structure labeled C in Plate IV, Fig. 11; Plate VII, Fig. 29 and structures labeled A and C in Plate IX, Fig. 36) or simply aligned in long chains (arrows in Plate XIV, Fig. 52).

Banding Patterns

Cross-striations appear on the immature SLS segments at approximately 35 seconds in the time-study as a very ill-defined band at one end of a fibril (see arrow at fibril A, Plate IV, Fig. 8). It is characteristic of the early stages of SLS formation that the banding pattern develops as a single cross-striation at one end of a segment (Plate IV, Fig. 8, fibril A; Plate V, Fig. 12, fibril A and Figs. 13 and 14; Plate VI, Fig. 15; Plate VIII, Fig. 27). This band is always found at the end of the SLS fibril which is most "mature"; that is, the end which most closely resembles the older SLS of classical description (compare the structure at the arrow, Plate V, Fig. 13 with Plate XI, Fig. 41 and Plate XIV, Fig. 52). Although developing cross-striations could be identified as early as 35 seconds in the time-study, the first easily recognized banding became evident at 60 seconds (Plate VI, Fig. 14) in this study.

As the SLS segments mature, banding can be seen at both ends of the fibrils, again as a single cross-striation (Plate VIII, Fig. 34; Plate XI, Fig. 40). Their earliest occurrence is at 140 seconds (Plate VIII, Fig. 34). They are prominent at 500 seconds (8 1/3 minutes; Plate XI, Fig. 41) and additional, less prominent bands, are evident. Several cross-striations are present at 9 1/3 minutes (Plate XII, Fig. 43) and a segment from a comparable time

period but with positive staining (Plate XII, Fig. 45) demonstrates their presence more dramatically.

The affinity of these preparations for the electron stains is quite variable, as demonstrated in Plate XII, Figs. 46 and 47. Both micrographs were taken from the same specimen screen, yet banding is evident in one and not the other.

The occurrence of these SLS segments as real entities and not as artifacts of the negative staining process was confirmed by using four other methods of preparation: shadow-casting with heavy metals (Plate XIII, Figs. 48 and 51), positive staining with phosphotungstic acid (Plate XII, Fig. 45; Plate XIII, Figs. 49 and 50; Plate XV, Figs. 53 and 54), methacrylate embedded and sectioned SLS (Plate XVI, Figs. 56, 57 and 58) and negative staining with osmic acid vapors (Plate XVI, Fig. 59).

The structures at the arrows in Plate XIII, Figs. 50 and 51, yield some interesting formation. The SLS fibril in Plate XIII, Fig. 50 appears to have formed adjacent to an electron-dense globular mass of unknown composition. Examination of the cross-striations (arrows) reveals them to be "deformed" or altered from their usual nearly perpendicular alignment with the long axis of a segment (compare with the segment in Plate XII, Fig. 45). The distortion of the band pattern occurs at only one end of the SLS and across only one-half its width. A similar deformation of the cross-striations is present in a shadow-cast preparation (Plate XIII, Fig. 51). The arrows point to the distorted band pattern at one end of a segment while the striations at the opposite end remain in their usual

relationships. This observation is inconsistent with currently accepted theories about the tropocollagen molecules and this point will be discussed in detail.

Embedded and Sectioned Preparations

An attempt was made to use sectioned material to confirm or disprove the results found by the previously described methods. The use of embedding and sectioning techniques on solubilized and reconstituted materials has not been described previously. The detail achieved by this method when it has been applied to solubilized and reconstituted collagen preparations is inferior to the detail found in sections of intact connective tissue or in the unembedded material. This lack of contrast is demonstrated for both carp SLS (Plate XVI, Figs. 56, 57 and 58) and native-type fibrils from rat tail tendon (Plate XVI, Fig. 60).

The carp SLS segment in Plate XVI, Fig. 56, is 2894 A long near the mid-point of its long axis, a value that correlates with the unsectioned SLS in the time-study. The profile is similar to the unsectioned fibrils also (compare with Plate XIII, Fig. 51 and Plate XIV, Fig. 52). The cross-striations that are usually seen in mature, unsectioned, positively-stained SLS are poorly demonstrated in sectioned material (compare with Plate XII, Fig. 45). Several structures of various sizes and shapes were seen in the embedded SLS preparations (Plate XVI, Figs. 57 and 58). Although these were seen as frequently as the longitudinal sections of SLS (Plate XVI, Fig. 56) it is not clear whether these represent cross-sections of SLS or other stages in the aggregation of the collagen molecule. More work should be done in this area.

Negative Staining with Osmic Acid Vapors

Cox, Grant and Horne (1967) have emphasized the usefulness of negative staining in making accurate measurements of SLS. They used phosphotungstate solutions, as we have, for this purpose. Another negative staining technique, proposed by Barland and Rojkind (1966), used the vapors of osmium tetroxide. The fibril in Plate XVI, Fig. 59 was stained by a modification of this technique and the ends of the SLS are very sharply defined. This staining technique may prove to be a useful adjunct to presently accepted methods when attempting to get accurate measurements of macromolecules and other small structures.

CHAPTER IV

DISCUSSION

This time-study is the first detailed morphological demonstration of early fibrillogenesis in reconstituting systems of SLS. It is clear from Plates I and II that the earliest aggregation products are globular rather than filamentous structures. As SLS formation continues over time, the globular nature of the segments becomes more and more obscured (Plates I through XV) yet extrafibrillar globules and rounded projections on the SLS persist even in the oldest preparations (Plate XIV). Olsen (1967b) shows micrographs of "mature" SLS produced from the alpha chains of codfish skin collagen that have very definite globular structures associated with otherwise well-defined SLS segments (see Figure 9, Olsen, 1967b). The presence of globular units in his SLS preparations (see Olsen, 1967a, Figure 5; 1967b, Figures 8 through 15) were completely ignored. Tromans, et al., (1963) published micrographs of native-type collagen fibrils which revealed globular structures by negative staining also.

Gross, Highberger and Schmitt (1954) and Schmitt, Gross and Highberger (1955) published micrographs of "spheroids" that formed in mixtures of ichthyocol and 0.05 per cent ATP (see their Plate IV, both articles) that were very similar to those globules seen in Plate I, Fig. 1 and Plate II, Fig. 2.

Gross, et al., were studying chromium-shadowed preparations while ours are negatively-stained. Gross and his coworkers interpreted the "spheroids" to be clusters of tropocollagen particles coordinated around the negative groups of ATP and noted that they had never been able to produce SLS or FLS from pure collagen solutions without the addition of some evocating agent. The presence of globular particles or "spheroids" in this early work has been ignored in subsequent reports, even by these authors (Gross, et al., 1954) who found similar globular structures when SLS was allowed to dissolve in acid solutions (Gross, Highberger and Schmitt, 1954, their Plate I, Figure 2). They also demonstrated "dense spheroids" in an hexagonal packing arrangement that formed within "cloudlike aggregates" of collagenic material; an SLS was seen within these "cloudlike aggregates" (Gross, et al., 1954, their Plate I, Figure 5). An even more dramatic example of this phenomenon is shown in our Plate IX, Fig. 35, structure A. Gross, et al., (1954) note that, "Randall has suggested that collagen fibrils may be composed of globular units about 640 A in diameter. The spheroids described herein have diameters of this order of size. However, no evidence has as yet been found that these actually form part of the collagen fibrils (though they may well be involved in the formation of the fibrils)."

Much of the electron microscopic work on both SLS and native-type collagen fibrils has been done on positively-stained material. Extrafibrillar globular structures are most frequently seen in material prepared by negative staining and shadow-casting techniques. This result can be explained by the nature of the technical manipulations involved. Specimens prepared by positive

staining are subsequently washed in distilled water, buffers, or weak acid solutions. Therefore, only those particles that adhere strongly to the supporting film remain; the rest are washed away. Negative staining and shadow-casting do not involve a washing step.

It might be argued that the staining method produces artifacts that we are interpreting to be intrinsic to the SLS structure. However, specimen screens were examined that had been treated exactly like the experimental material, minus the stock ichthyocol solution, and no such globular units were found. These preparations included screens with supporting film only, film plus ATP, film plus stain, and film plus ATP and stain. Therefore, the structures observed must be intrinsic to the reconstituting collagen system.

The rounded projections that are frequently observed on SLS fibrils are present on positively stained segments (Plate XIII, Fig. 50) as well as those visualized by other methods (Plate XIII, Fig. 51) and in native-type fibrils (Plate XVI, Fig. 60) providing additional evidence that there is an intrinsic globularity to collagen fibrils of all types. Olsen (1967b, see his Figure 10) shows an SLS fibril with a splayed end and intrinsic globularity like that in Plate VI, Fig. 20 but by positive staining. Figure 9 in the same paper (Olsen, 1967b) reveals a micrograph containing fan-shaped SLS with globules associated, similar to Plate XIV, Fig. 52.

Having established that globular structures do, in fact, exist in SLS and other types of collagen preparations, perhaps we should now look at their possible role in fibrillogenesis and their relationship to the morphology of the

collagen molecule. The generally accepted theory assumes that the band pattern of SLS represents a "molecular fingerprint" of a fundamental monomer of collagen (Hodge and Schmitt, 1960; Hodge and Petruska, 1962, 1963; Petruska and Hodge, 1964; Hodge, et al., 1960; Cox, et al., 1967) named "tropocollagen" by Gross, Highberger and Schmitt (1954). From its physicochemical properties, the tropocollagen molecule is thought to be a long, stiff, rod-shaped macromolecule about 14-18 A in diameter, 2720 to 3500 A long and molecular weight 300,000 (Gross, et al., 1954; Jackson, 1958; Boedtker and Doty, 1954, 1955, 1956; Hodge and Schmitt, 1960). Cox, Grant and Horne (1967), however, state that, "Although, physicochemically, the tropocollagen macromolecule is regarded as a rigid rod, morphologically, it possesses some flexibility" and that, "the part played by the long linear protofibril in the formation of the native-collagen fibril ...has been too readily accepted." Veis, et al., (1970) also suggest that a complication of physicochemical studies is "... the probability that very long thin rods might behave as flexible structures rather than rigid ones." No such long, rod-shaped structures are evident in published micrographs of collagen.

Electron microscopic studies of shadowed preparations of solubilized collagen have yielded pictures of long, filamentous units which were interpreted to be the "tropocollagen" molecule (Hall, 1956; Hall and Doty, 1958; Rice, 1961). Examination of those published micrographs, however, reveal a "beaded" pattern along the "filaments" plus globular particles of the same size as the "beaded" elements scattered among the filamentous structures. An earlier

paper by Porter and Vanamee (1949) reported beaded filaments in chick embryo skin explants that were 50-100 A in diameter with a distance between "beads" of 270 A and a periodicity of 270 A. Keech (1954, 1955) treated human skin and tendon and calf tendon with collagenase and found fibrils with tapered ends and/or localized narrowing of fiber width plus tactoids (short lengths of collagen tapered at both ends). Beaded fibrils and isolated globules were present in her preparations, as they were in preparations from bovine skin studied similarly by Gross (1953). Tactoids, whose tips usually end in tiny globular structures, have been noted by Noda and Wyckoff (1951), Banfield (1952, 1955), Randall, et al., (1955), Randall (1957) and Gross (1953). Keech (1954) reported that the beaded filaments and tactoids were frequent in infant skin but occurred only occasionally in adult skin. Banfield (1952, 1955) also noted the tapered fibrils most frequently in embryonic connective tissue and in human mesenchymal neoplasms. Curran and Clark (1963) produced avascular granulomas on peritoneal surfaces and found that early fibrogenesis was characterized by the presence of beaded filaments less than 50 A in diameter in the vicinity of each fibroblast. Beaded filaments consisting of paired 40 A strands in parallel coupled by 150 A beads at periodic spacings of 640 A have been isolated from embryonic pig dermis by Hayes and Allen (1967). The formation of tactoids have been found to vary with salt concentration and time (Vanamee and Porter, 1951). Borasky and Shimony (1965) found transverse cleavage planes and other structural defects in bovine skin collagen that occurred at macroperiod levels and suggested that the collagen molecule was about 650 A

long rather than 2720 to 3500 A long.

These reports resemble our findings that the early stages of SLS fibrillogenesis are characterized by globular structures while the latter tend to become obscured in the mature SLS preparations.

During the preparation of this report, a paper was published by Veis, et al., (1970) on the solubilization of mature, polymeric collagen fibrils by lyotropic relaxation, i.e., the swelling and dispersion of collagen by using chelating agents such as EDTA. The results were interpreted to be an effect caused by the "lyotropic relaxation" process, in which "microfibrillar filaments" with diameters less than 100 A were thought to be visualized. Their Figures 4, 5 and 6, however, are almost identical to some of the SLS forms produced during our time-study, in which no chelating agent or denaturing process was used (see Plates III, IV, VI, VII, VIII, IX, XI and XII, Fig. 45). The magnification used in their study (Veis, et al., 1970) was too low to allow detailed examination but globular structures were evident, nevertheless, in association with the SLS and in the extrafibrillar material.

The length of SLS fibrils is currently assumed to be that of the "tropocollagen" particle (Olsen, 1963b; Hodge and Schmitt, 1960; Gross, Highberger, and Schmitt, 1955; Cox, Grant and Horne, 1967) and various hypotheses have been advanced to explain the lateral aggregation of long, stiff, rod-shaped macromolecules and the band patterns that are seen in native-type fibrils, FLS and SLS (Schmitt, Gross and Highberger, 1955; Gross, 1956; Schmitt, 1956, 1959, 1963, 1964; Hodge and Schmitt, 1961; Hodge and

Petruska, 1963; Olsen, 1967; Cox, Grant and Horne, 1967). No such rod-shaped molecule has been identified in SLS or any other collagenic material and the proposed molecular packing arrangements do not adequately explain the banding patterns that have been observed. Hodge and Petruska (1962) found that the proposed length of the tropocollagen molecule and the length of an SLS segment exceeds the length of four periods of native-type fibrils (i.e., greater than 2560 A). Gross, Highberger and Schmitt (1954) and Schmitt, et al., (1955) reported that SLS range in length from 1500 to 3000 A. Kuhn, Hoffmann and Grassmann (1959) found a range of 2400 to 3200 A, while Olsen (1963b) reported an average length of 3130 A with a range of 2720 to 3400 A. This study demonstrates a variation from 1889 A to 4378 A with an average of 3010 A, or a difference of 2489 A between the longest and shortest fibrils recorded. The length of the tropocollagen molecule, on the other hand, has been recorded to be 500 to 4000 A, average 2200 A, by electron microscopic studies (Hall, 1956) and 2720 to 3500 A with a range of 2720 to 160,000 A by physicochemical techniques (Bailey, 1968; Bear, 1942, 1952, 1955, 1957; Boedtker and Doty, 1955, 1956; Engel, 1962; Harrington and von Hippel, 1961; Rice, et al., 1964; von Hippel, 1967), demonstrating considerable variation between observed lengths of SLS and reported lengths for the tropocollagen molecule. This time-study has demonstrated a difference between the longest and shortest SLS fibrils measured (4378-1889 = 2489 A) that approaches the SLS lengths reported by other investigators for mature SLS. Alternative proposals for the structure of the

monomeric unit of collagen present even greater variation between the length of the suggested collagen molecule and SLS aggregates (Porter and Vanamee, 1949; Randall, 1953; North, Cowan and Randall, 1954; Cowan, North and Randall, 1955; Kahn, Carroll and Witnauer, 1962; Borasky and Shimony, 1965; Davidovits, 1966; Richter and Schilling, 1966; Veis, et al., 1967; Bhetnager, et al., 1968).

The tropocollagen theory also does not account for the great variability in periodicity of native-type fibrils: 400 to 1000 A (Gross, et al., 1954); 520 A (Banfield, 1952); 300 to 1000 A (Schmitt, Hall and Jakus, 1942); 210 A (Vanamee and Porter, 1951); 210 A and 640 A (Keech, 1955); 270 A (Ornstein, 1956); 420 A (Richter and Schilling, 1969); no striations in earthworm cuticle collagen (Maser and Rice, 1962); 220 A in embryonic tissues (Gross, 1956); and variations of 220 A., 640 A or no periodicity in reconstitution studies, all being interconvertible (Gross, 1956). Schwartz, et al., (1969) found that stretching and compression of collagen varied the lengths of the periods.

Comparatively little attention has been given to the widths of SLS and their relationship to the possible packing arrangement of tropocollagen or other proposed collagen molecules within the fibrils (Gross, et al., 1952; 1954; Schmitt, et al., 1955; Kuhn, et al., 1959; Hodge and Petruska, 1962; Olsen, 1963b). One would assume that a side-by-side aggregation of cylindrical macromolecules would yield SLS, and other types of fibrils, whose widths would be relatively uniform along the length of the fibrillar aggregate. This time-study of SLS fibrillogenesis, however, reveals significant variation in the widths of the

segments at different points in the same segment (see Plate III, Fig. 6; Plate IV, Fig. 8, fibril labeled B; Plate V, Fig. 13; Plate VIII, Fig. 27; Plate X, Fig. 38; Plate XI, Fig. 40; Plate XII, Fig. 43; Plate XIII, Figs. 49 and 51; Plate XIV, Fig. 52). The data for the averages and ranges of widths at each time period (Table 2 and Graphs 3 and 4) show that the difference between the narrowest point recorded for the "narrowest point" width (92 A) and the narrowest point recorded for the "widest point" measurement (276 A) was 184 A. That is, the least value found in the widest point data was three times the narrowest width recorded in this study (Table 2, Range). The data for the four time periods in which 92 A was the least width recorded (Table 2 at 20, 25, 40 and 100 seconds) reveal an even greater difference between the "narrowest point" and the "widest point" measurements (599 A - 92 A at 20 seconds; 415 A - 92 A at 25 and 40 seconds; 461 A - 92 A at 100 seconds). There is no way to account for these results if one accepts that the collagen molecules are cylinders 14-18 A in diameter by 3000 A long (or any other length) and aggregate side-by-side.

Development of Band Patterns

Cross-striations in collagen were first described by Schmitt, Hall and Jakus (1942) and the conventional labeling was proposed by Schmitt and Gross (1948). The bands of collagen fibrils were defined by Bear (1942) as "disk-like enlargements of fibrillar diameter with greater than average power to deflect or absorb electrons and to take up electron stains. The bands form characteristic patterns which periodically repeat along the fibril axis and which are thought to

be regions of charged side-chains which absorb anionic and cationic electron stains. The fibrillar levels remaining between bands may be called "interbands." The latter have been thought to contain primarily non-polar residues and therefore to poorly absorb electron stains (von Hippel, 1967). According to the concept elaborated by Schmitt (1956), periodic banding of fibrils, or cross-striations, occur when a substantial fraction of the amino acids in a protein possess long side-chains which give rise to lateral interactions between macromolecules, thereby yielding regions of relative order and disorder, depending on the way the side-chains adjust to the closely packed fibrous configuration. These ordered and disordered regions were thought to occur at precise locations along the fibril and to reflect the distribution of the side-chains along the length of the fibril. Since the amino acid distribution is supposed to be constant and specific for each protein, lateral interaction between side-chain groups of the macromolecules should form fibrils which appear banded in the electron microscope.

This time-study has demonstrated, however, that cross-striations are a relatively late appearing structural feature (see arrow at fibril A in Plate IV, Fig. 8 and in Plate V, Fig. 12; see Plate V, Figs.13 and 14; Plate VI, Fig. 15; Plate VIII, Fig. 27). One band characteristically forms first at one end of a fibril (Plate IV, Fig. 8); later in fibrillogenesis an additional single band develops at the opposite end of the SLS (Plate VIII, Fig. 34; Plate XI, Fig. 40), the earliest occurrence of two recognizable bands being at 140 seconds in this study. The SLS segments are relatively old before several bands are evident (Plate XII, Fig. 43 at 9 1/3 minutes; Plate XII, Fig. 45).

It would seem that cross-striations should develop as rapidly as the SLS form if the concept of the lateral aggregation of tropocollagen molecules is correct. The currently accepted concept that the three polypeptide chains of collagen are formed and interact to form a triple helix, the tropocollagen molecule, prior to their lateral aggregation into native-type fibrils, FLS or SLS, suggests that the side-chains assumed to be responsible for the banding patterns are present and available for staining at the time of fibril formation. The relatively slow development of cross-striations over time in SLS fibrillogenesis cannot be accounted for by this theory.

The concepts that have developed concerning the tropocollagen molecule and its aggregation to form various types of fibrils and cross-striations (Gross, Highberger and Schmitt, 1954; Schmitt, et al., 1955; Boedtker and Doty, 1955; Hall, 1956; Schmitt, 1956; Jackson, 1958; Hall and Doty, 1958; Hodge and Schmitt, 1960, 1961; von Hippel, 1967) also do not explain the occurrence of the "deformed" band patterns demonstrated in Plate XIII, Figs. 50 and 51. The currently accepted theories suggest that if the cross-bands at one end of an SLS, or any type of fibril, are deflected in the direction of the long axis of the fibril (Plate XIII, Fig. 50) then all the bands along that side of the segment should be similarly deflected out of their normal configuration, i.e. nearly perpendicular to the long axis of the fibril (Plate XII, Fig. 45). Examination of Plate XIII, Figs. 50 and 51, reveals that this is not the case. The fact that a similar deformation of the usual band pattern has been demonstrated by two completely different techniques, suggests that a different interpretation of macro-

molecular aggregation and band pattern development must be formulated. This time-study did not provide the necessary data for such theorizing. However, a new concept of the structure and packing arrangement of the collagen macromolecule has been developed by Doctor Kenneth M. Richter at the University of Oklahoma Medical Center, based on the visualization of a pear-shaped structure in electron micrographs of several types of connective tissue (Richter and Schilling, 1969, 1970). An extensive correlation of electron microscopic, physicochemical, x-ray diffraction and biochemical data has recently been completed by Richter and will be published in the near future (Richter and Schilling, 1970, *personal communication*). Kenneth M. Richter's concept of a pear-shaped collagen molecule and the mechanism of aggregation that he is proposing is the only hypothesis to date that accounts for the length and width data, development of band patterns and intrinsic globularity of SLS found in this study.

Embedded and Sectioned Preparations

There are no previous reports in the literature on reconstituted collagens that describe the findings in embedded and thin-sectioned material, be it SLS, FLS or native-type fibrils. We have used this technique but the difficulties encountered in attempting to stain embedded and sectioned specimens of reconstituted collagens (SLS and native-type fibrils) and a limited amount of time available for the study led us to abandon this approach. Therefore, our observations can be reported only as preliminary results that will require more extensive study.

The problems associated with staining this type of preparation (see Plate XVI) might possibly be explained by a relative absence of bound ionic

groups on this material that could react with the heavy metals in the staining solutions. Specimens prepared by simply precipitating fibrils directly on grids are stained prior to any washing step. Material to be embedded is first fixed, which would tie up many reactive groups on the fibrils, and then undergoes dehydration and embedding through a series of fluids that could remove loosely associated or unbound ionic groups and salts (compare Plate XIII, Figs. 49 and 50 with Plate XVI, Figs. 56, 57, 58 and 60). It is thought that electron stains normally react with bound ionic groups in the unembedded preparations of reconstituted collagen and that fewer such groups are available in fixed and sectioned material. The absence of this staining difficulty in most standard connective tissue sections might be explained by the presence of mucopolysaccharides and other high molecular weight ionic compounds associated with it and not washed away by the processing fluids. Protein-polysaccharide complexes have frequently been described in association with collagen fibrils. Meyer (1947) suggested that collagen is formed on a mucopolysaccharide template. Smith and Serrafini-Fracassini (1968) demonstrated a protein-polysaccharide complex associated with fine collagen fibrils in the matrix of nucleus pulposus and in cartilage. Schwartz (1961) found hyaluronate attached to fine collagenous filaments in the vitreous body and Myers, Highton and Rayns (1969) found acid mucopolysaccharides to be closely associated with collagen fibrils in normal human synovium. Theoretical support for this hypothesis may be found in the discussion of osmic acid negative staining by Barland and Rojkind (1966). Robertson (1964) stated that, "The behavior of collagen, a polyelectrolyte, is modified by other poly-

electrolytes commonly associated with it, e.g. mucopolysaccharides and (muco)-proteins, by smaller ions, and even by such non-electrolytes as urea." As noted in Chapter III, Observations, however, the lengths and the profile of sectioned SLS correlate well with the results found for the unembedded materials.

Negative Staining with Osmic Acid Vapors

Various authors have raised the question of the accuracy of length measurements in negatively stained versus positively stained SLS (Cox, Grant and Horne, 1967; Olsen, 1967). The negative staining technique yields length values for SLS that are 10% greater than positive staining (Olsen, 1967) and Cox, Grant and Horne (1967) considered the negative staining method to be the more accurate of the two. Solutions of phosphotungstic acid are generally used for this purpose. Negative staining with the vapors of osmium tetroxide was described by Barland and Rojkind in 1966. A slight modification of this technique has been used on various types of collagen preparations in our laboratories with some success. Plate XVI, Fig. 59 demonstrates the sharp boundaries of an SLS fibril that can be outlined by this method. We therefore suggest that future attempts to measure the dimensions of SLS or other macromolecular aggregates may profitably utilize this technique.

CHAPTER V

SUMMARY

It has been suggested and generally accepted that attenuated collagen molecules in solution (14-18 A diameter by 3000 A length) are aggregated in a side-by-side alignment, without change in size and shape, into native-type FLS and SLS forms of reconstituted collagen. It has been assumed that the cross-banded SLS form represents, from one end to the other, a "molecular fingerprint" of the solubilized collagen molecule.

To evaluate this hypothesis the present study was undertaken to obtain morphologic and quantitative data on the genesis of collagen complexes, particularly the SLS form using an in vitro reconstituting system.

The bulk of the data obtained is based on solubilized collagen extracted from carp swim bladder; the remainder concerns solubilized collagen from rat tail tendon, dog tendon and skin and induced fibrocollagenous cylinders from the dog.

Morphologic data show that all reconstituted collagens (native-type, FLS and SLS) from several sources have an intrinsic globular structure. Time-sequence studies (5 seconds to several minutes of reconstituting carp swim bladder collagen yield the following information on SLS formation.

(1) A fine globular structure characterizes all stages in the genesis of

SLS, from the least to the most highly differentiated stages.

(2) The definitive cross-banding pattern of reconstituting SLS forms is time-dependent. Cross-bands appear first only at the ends of the SLS segments; the centers become progressively more striated over time.

(3) The lengths of reconstituting SLS vary with time and are also variable both within the limits of one SLS segment and from one segment to another (e.g. 1824 A to 2292 A).

(4) The widths of reconstituting SLS vary with time; they also vary within the limits of one SLS and from one segment to another.

(5) The cross-banding pattern is subject to mechanical deformations that are inconsistent with currently accepted theories of collagen ultrastructure.

The morphologic and quantitative data presented on the emerging segment-long-spacing type of collagen is not consistent with the hypothesis that fibrillogenesis involves a side-by-side alignment of structural subunits possessing the dimensions of a long, rod-shaped macromolecule. Rather, the structural subunit involved is of a globular form.

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APPENDIX

PLATE I

Figure I. Carp SLS at 5 seconds in the time-study. Note the many globular forms of various sizes and the bead-like ultrastructural detail common to all the globular units and filamentous structures. The tiny filament at A is approximately 18 A in diameter and the beaded structure at B is 45-65 A in diameter. A coiled figure occurs at C composed of a central sphere, 74-92 A diameter, with an extended filament of similar dimensions coiled around it. There is a pear-shaped structure at D that is 275 to 295 A long, about 92 A wide at its narrow end and about 148 A wide at the bulbous end. The SLS-like segment (arrow) in this micrograph is the only one observed at this period in the time-study. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

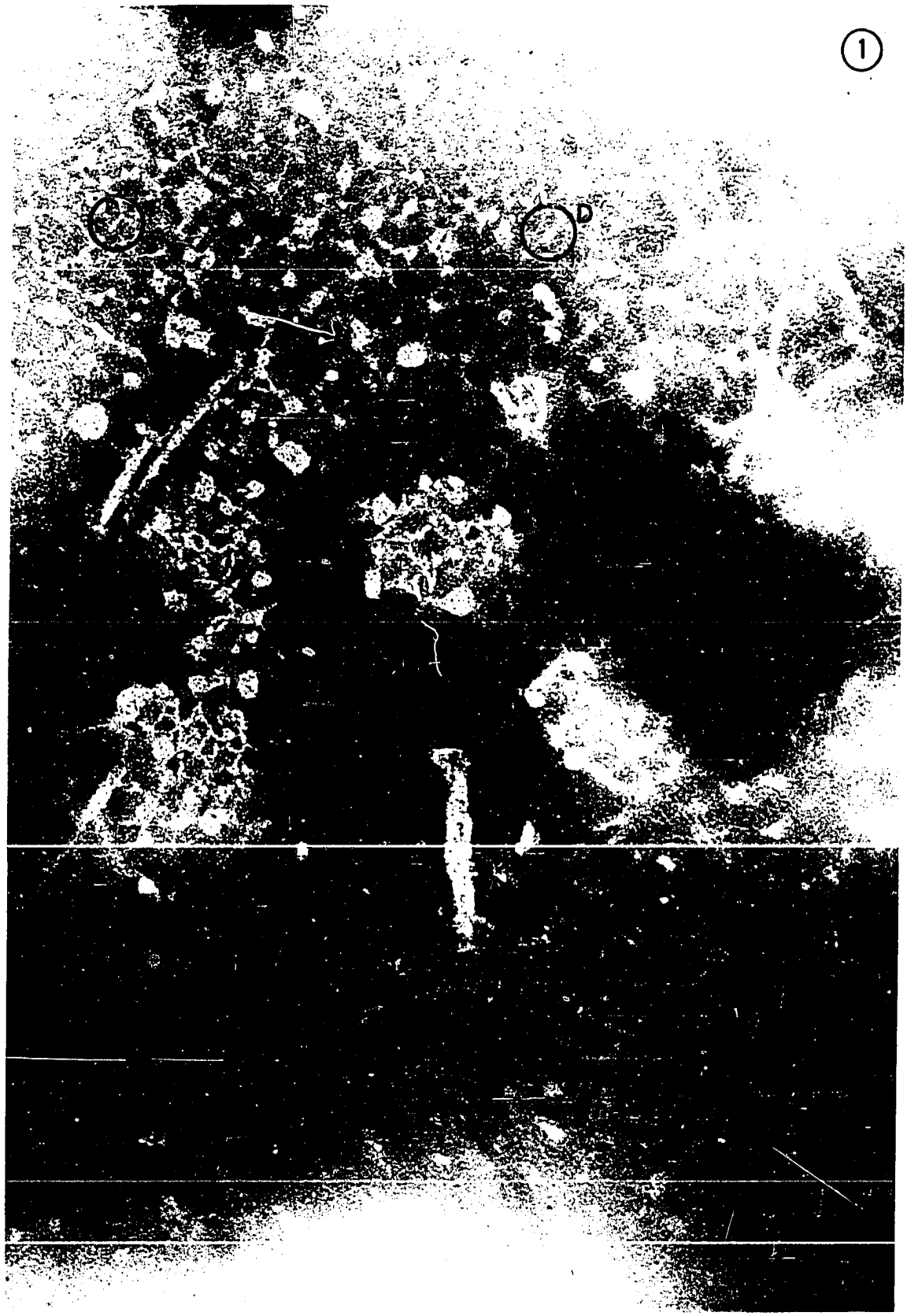
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PLATE II

Figure 2. Carp SLS at 10 seconds in the time-study. Globular units predominate not only as discrete units but as parts of extended SLS-like fibrils at A. A suggestion of banding appears in the irregular, elongated structure at B. The ultrastructure is the same in the globules and SLS-like fibrils as in Plate I. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 3. Carp SLS at 15 seconds in the time-study. The fibrillar elements have become much more uniform and SLS-like in appearance, yet rounded projections occur along the borders of segments (A) and globules are present in the extra-fibrillar areas. One end of the fibril at B is rounded. There is considerable variation in the overt lengths of the various fibrous structures. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108, 500.

Figure 4. Carp SLS at 15 seconds in the time-study. Note the significant variation in the length of this segment along the border labeled A compared with the side labeled B. Much of the filamentous material in the so-called extra-fibrillar area appears to be continuous with the fibril. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 5. Carp SLS at 15 seconds in the time-study. The immature SLS segment at A is similar in shape to the one in Figure 4, but is shorter by 900-1000 A. The segment at B has an irregular stair-step profile similar to the immature collagen fibrils described by Richter and Schilling (1970) in their sectioned scar material. Bulbous projections are present along its lateral borders and the width varies significantly along the length. The irregularly shaped structure at C has the same bead-like ultrastructure seen at all stages in the developing SLS, yet its length and shape are not typical of a classical SLS segment and one end appears to be continuous with the unorganized extra-fibrillar collagenic material. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

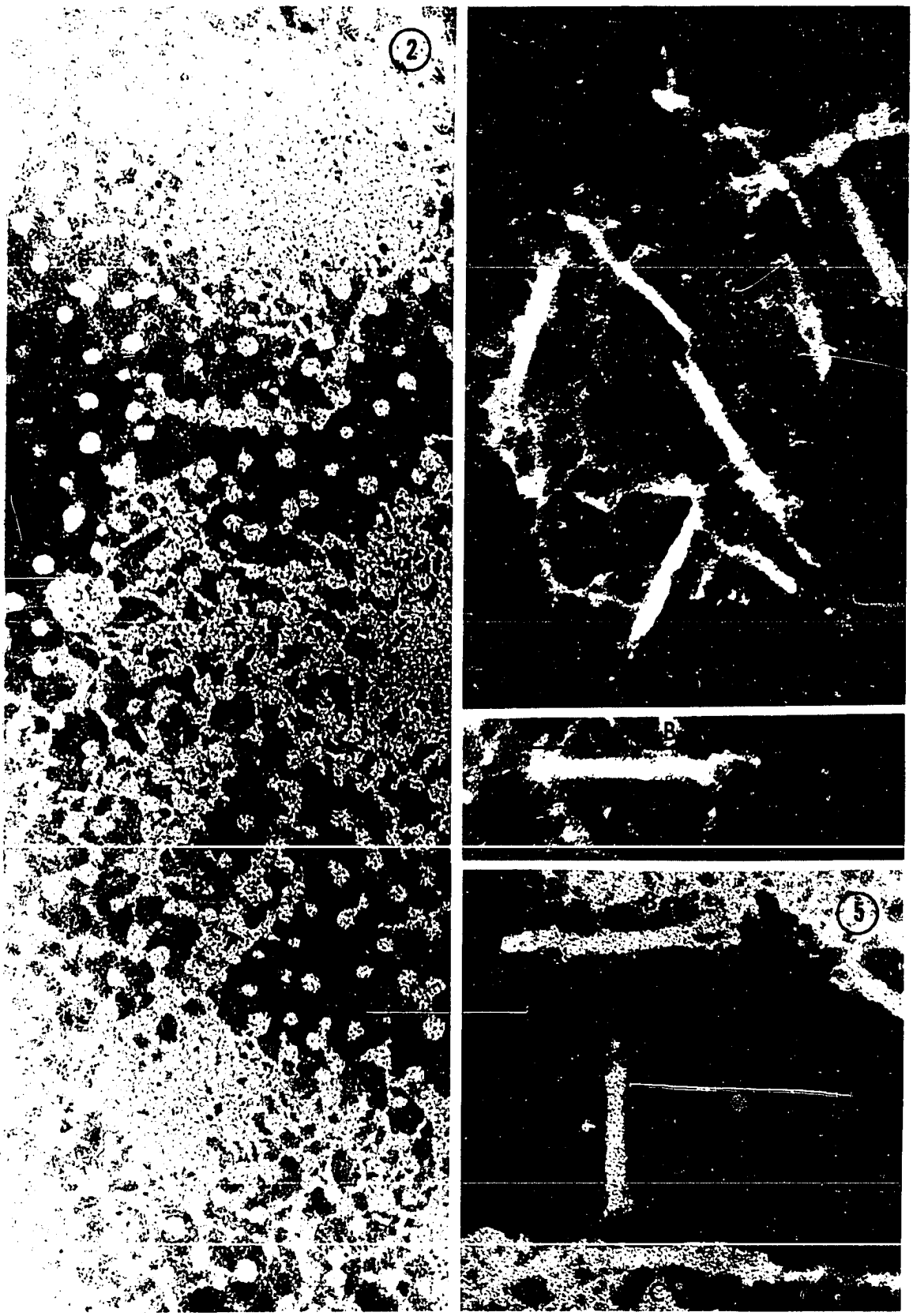
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PLATE III

Figure 6. Carp SLS at 20 seconds in the time-study. This micrograph demonstrates the variability in shape that occurs in SLS, especially in the early stages of formation. The splayed ends of the segment at A reveal both globular and fibrillar elements, but even the latter contain a bead-like globular ultrastructure. These SLS vary tremendously in width along their long axes. The segment at B is quite uniform at one end but is very irregular at the opposite, displaying a variation in the length of the segment, at different points across its width. This is even more evident at C where the stair-stepping effect in the profile resolves into two projections at one end. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 7. Carp SLS at 25 seconds in the time study. The segments at this time period are more uniform in size and shape than those at earlier stages. The splayed ends of the segment at A reveal globular units similar in size and shape to those in the extra-fibrillar material (arrow). Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

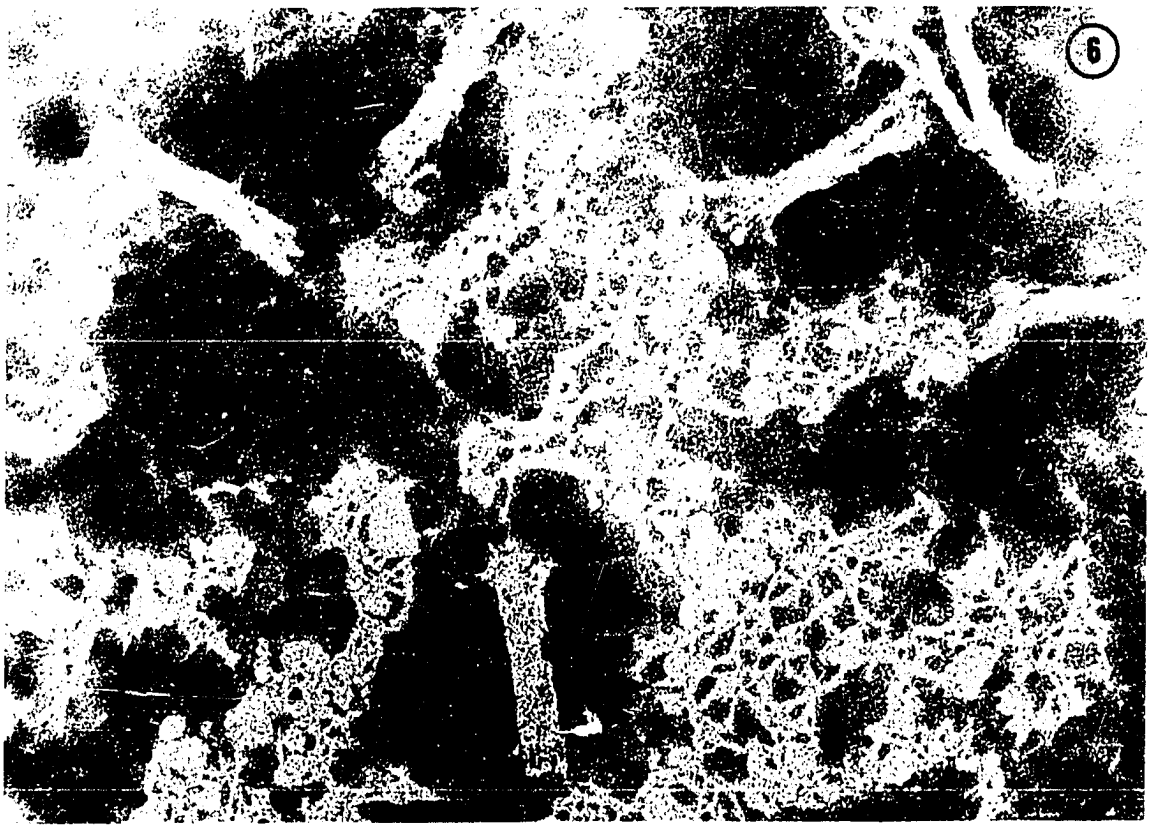
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PLATE IV

Figure 8. Carp SLS at 35 seconds in the time-study. There is a discrete and relatively uniform SLS at A which demonstrates an early appearance of banding (cross-striations) at one end (arrow). The structure at B has relatively large globular units, similar to the globules in the extra-fibrillar material, projecting from one end. The large masses of electron dense material appear to be aggregates of the globular electron dense material. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 9. Carp SLS at 35 seconds in the time-study. This segment is very irregular at one end and appears to be continuous with globular units, yet an early cross-striation is present in this fibril. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 10. Carp SLS at 40 seconds in the time-study. Note the globular appendage projecting from the side of this segment. It has the same bead-like ultrastructure as the main fibril. The latter appears to have three of these larger globules lined up across one end; the opposite end tapers to an irregular terminus composed of the same bead-like elements (45-65 A in diameter). One lateral border appears to be connected to the irregular, unorganized extra-fibrillar material. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 11. Carp SLS at 45 seconds in the time-study. The globular substructure of the immature SLS and the extra-fibrillar substance is readily apparent in this micrograph. The segment at A terminates in a globular structure measuring approximately 138 A by 203 A. Structures measuring about 45 A by 138 A are evident throughout the micrograph and appear to comprise the substructure of larger globular units (C). Some of these small units appear to project from the lateral margins and ends of SLS-like segments (B). Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

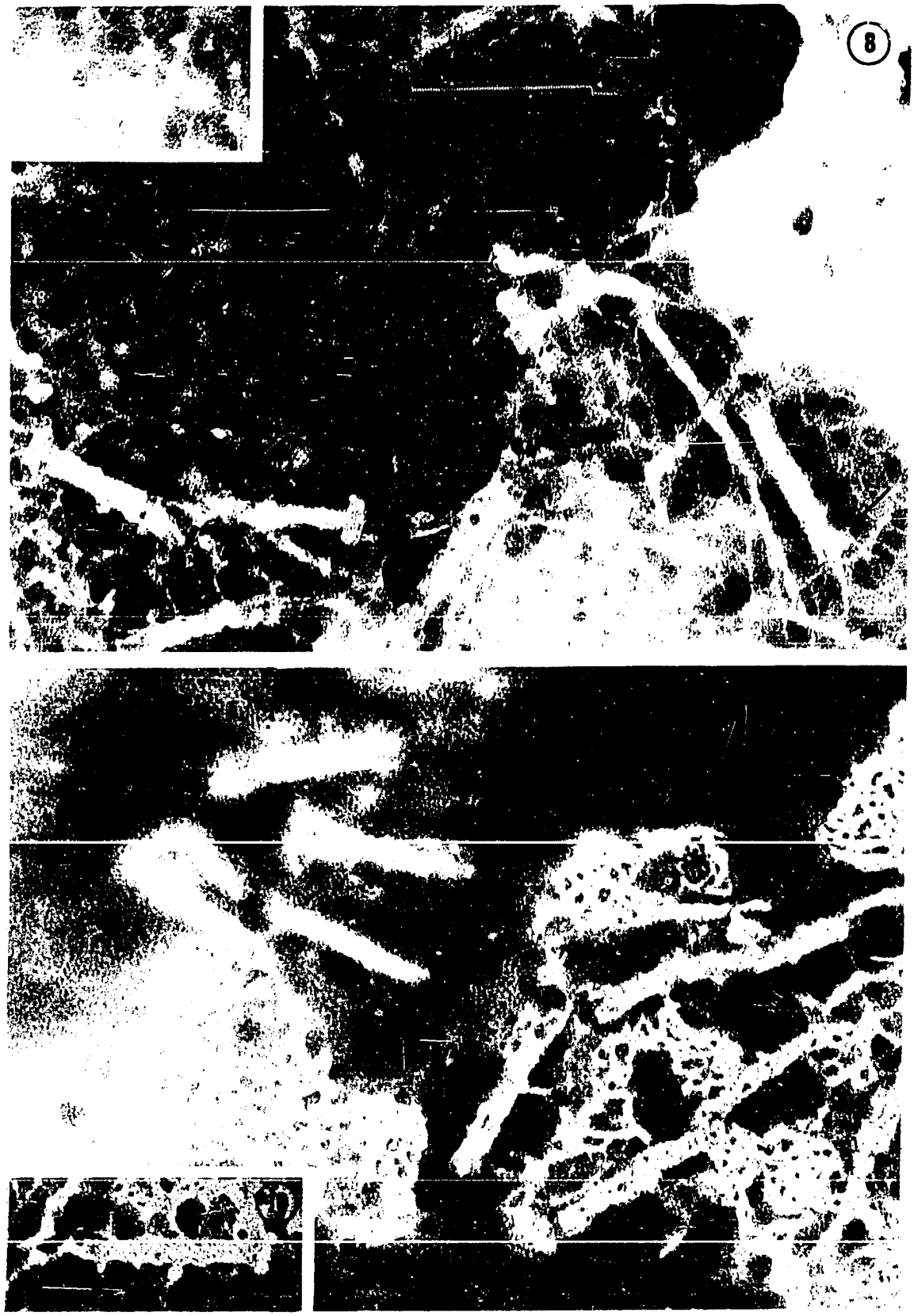
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PLATE V

Figure 12. Carp SLS at 50 seconds in the time-study. The extra-fibrillar material in this micrograph appears to be more fibrillar than globular in configuration; globules are present, however, and they occur as parts of the immature SLS (A) as well as in the extra-fibrillar substance (C). Large "spheroids" (691 to 829 A diameter) are present in the extra-fibrillar material and apparently as a part of fibrils (B). Cross-striations are present at the ends of segments (arrows). Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 13. Carp SLS at 55 seconds in the time-study. Globular elements of various sizes are present in the extra-fibrillar areas and in association with the SLS fibril. The latter terminates in a globular structure that is 184 to 203 A in diameter. At the opposite end of the segment are filamentous structures with a bead-like ultrastructure. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 14. Carp SLS at 60 seconds in the time-study. A definite cross-striation is present in this SLS segment which has a projection at one end. The smallest discernible filaments are about 18 A in cross-section. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

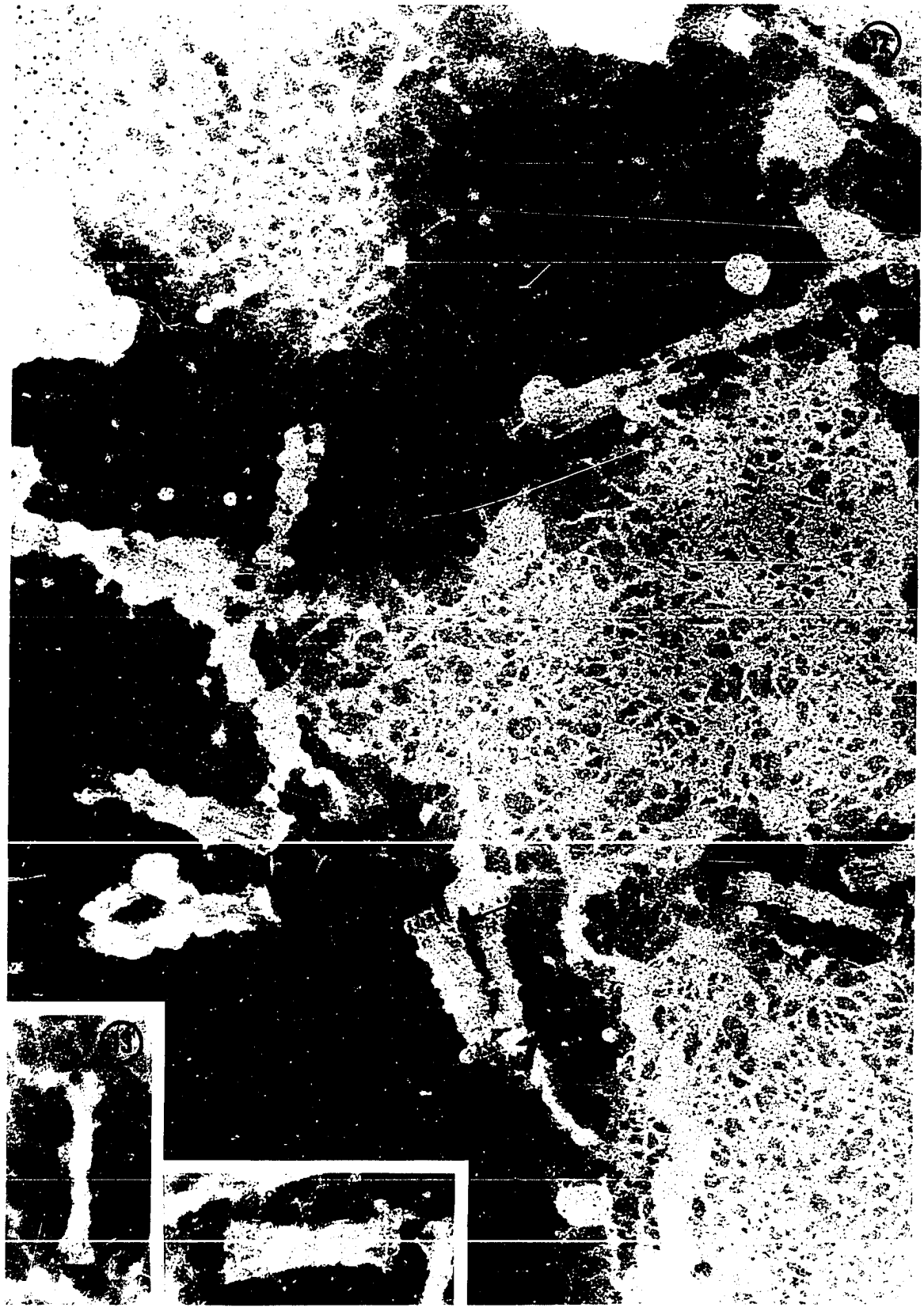

PLATE VI

Figure 15. Carp SLS at 70 seconds in the time-study. There are numerous globular units in this micrograph and many appear to be associated with immature SLS segments (A). Banding is present in the more uniform SLS (arrow) and is most distinct at one end only. The filaments at the ends of the segment at B contain bead-like globules, 45-65 A wide. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 16. Carp SLS at 70 seconds in the time-study. This fibril has a rounded end that is about 700 A wide composed of 45-65 A globular subunits. Larger globules are apparent in the extra-fibrillar area. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 17. Carp SLS at 80 seconds in the time-study. The extra-fibrillar material appears more filamentous with fewer large globular elements. Close examination reveals tiny globular units within the filaments, however. One end of each SLS segment is often quite uniform in structure, but the other end is frequently irregularly "frayed" or bulbous in profile. The little globular unit in the segment at A is about 100 A in diameter. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 18. Carp SLS at 80 seconds in the time-study. Irregular aggregates of globular units of various sizes are frequently seen in this time study, as demonstrated here. The ultrastructure is the same as the more typical SLS, however, since the bead-like globular elements are present in both. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 19. Carp SLS at 80 seconds in the time-study. This figure came from a specimen area containing many relatively uniform SLS segments having the same bead-like ultrastructure with the 45-65 A globular units; the latter are continuous in this figure with long beaded filaments at one end that give the segment a frayed appearance. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 20. Carp SLS at 80 seconds in the time-study. This is an example of the apparent "fraying" that occasionally is noted in an otherwise typical SLS segment. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

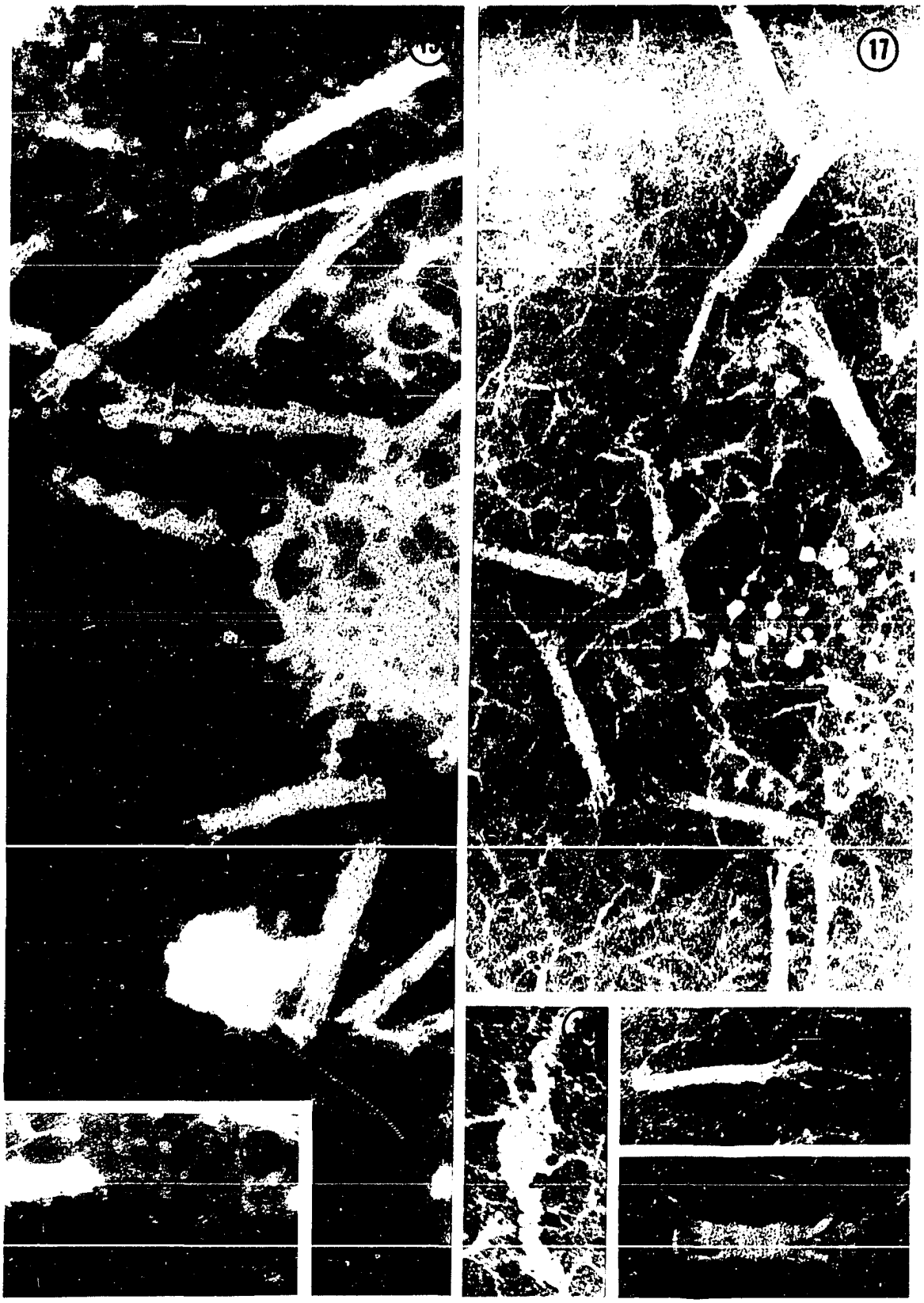
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PLATE VII

Figure 21. Carp SLS at 90 seconds in the time-study. The globular ultrastructure of the SLS fragments and the extra-fibrillar material is evident. The dimensions of these globular units (45-65 A) are the same at one and one-half minutes as they were in the earliest stages of SLS development. Larger globular structures are also present. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 22. Carp SLS at 90 seconds in the time-study. This demonstrates the apparent aggregation of 184 to 277 A diameter globular units that are frequently observed in these specimens of immature SLS fibrils. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 23. Carp SLS at 90 seconds in the time-study. These two SLS segments appear to be forming within a "cloud" or background of globular units. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 24. Carp SLS at 100 seconds in the time-study. Globular units 230-300 A diameter associated with the fibril at A have the same globular ultrastructure as the globules in the extra-fibrillar material (C). The segment at B demonstrates the variation in length that occurs in many immature SLS at different points across their width; this fibril terminates in a globular unit that is 74-92 A in diameter. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 25. Carp SLS at 100 seconds in the time-study. Note the variations in density that occur along the length and width of an immature SLS segment. The tiny globular units at the ends of this fibril do not appear to be packed as tightly as those at the center. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 26. Carp SLS at 100 seconds in the time-study. This micrograph demonstrates an unusual curved configuration in a segment that has the same size and ultrastructural detail as the other immature SLS fibrils. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

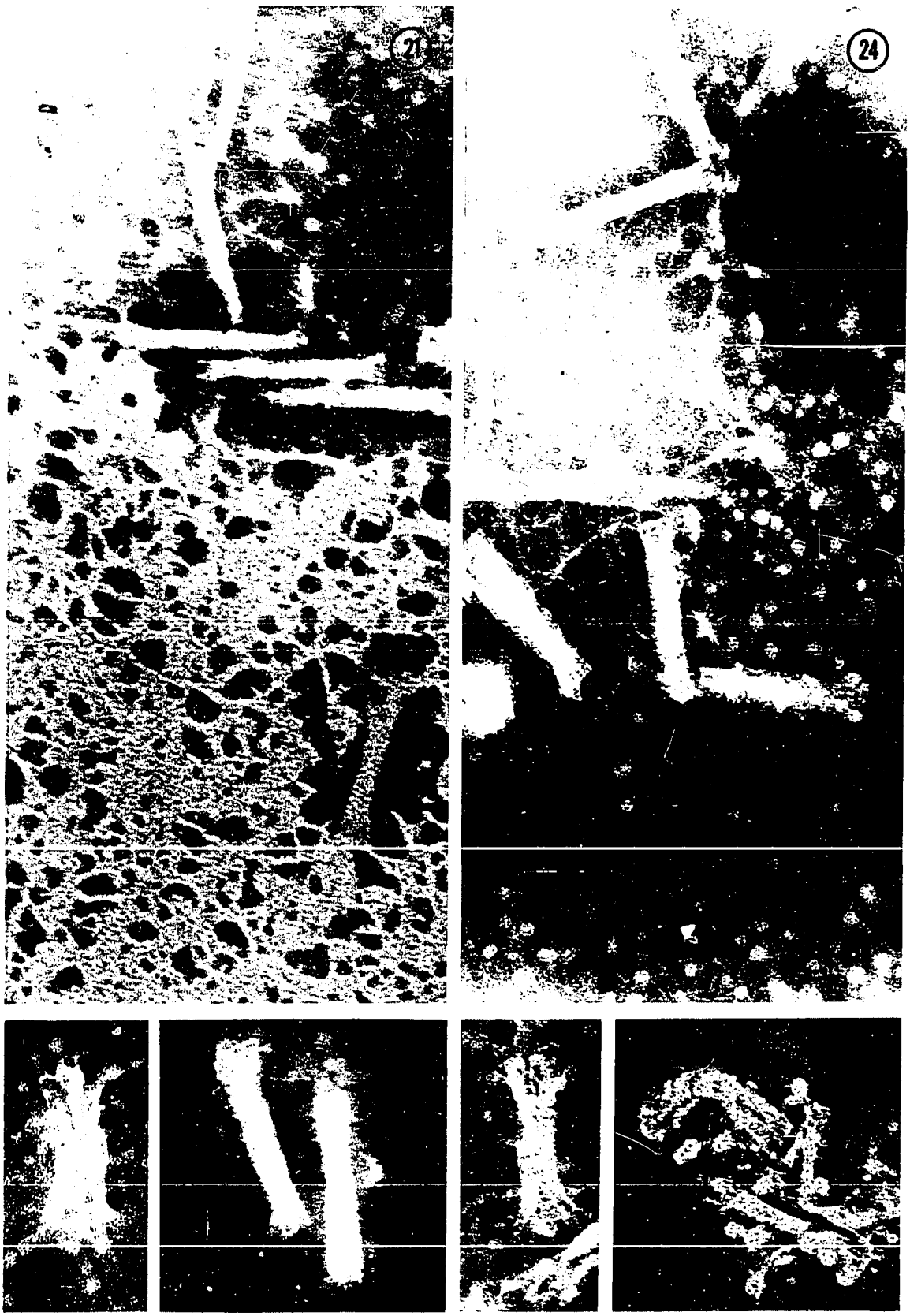
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PLATE VIII

Figure 27. Carp SLS at 110 seconds in the time-study. Note the bulbous or globular units projecting from the lateral edges of these fibrils and forming one end of the smaller segment. Similar globules are present in the extra-fibrillar area. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 28. Carp SLS at 110 seconds in the time-study. This segment has globular elements at each end as well as along its margins and demonstrates the stair-step effect noted previously in the immature SLS and described in immature native collagen fibrils by Richter and Schilling (1970). Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 29. Carp SLS at 110 seconds in the time-study. Several relatively discrete globular elements are aligned in linear array. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 30. Carp SLS at 120 seconds in the time-study. The fibrillar segments formed by two minutes demonstrate fragments that appear to be continuous along one-half the width of one segment (A) but not its other half. Several segments (A,B,C,E) reveal filamentous projections from their margins; these filaments have a globular ultrastructure. There is a rather diffuse unstructured end on the segment at C. Isolated globular units are apparent at D and the segment at E has an irregularly shaped terminal appendage. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 31. Carp SLS at 140 seconds in the time-study. The racket-shaped segment in this micrograph is very different from previously published pictures of SLS, yet it has the same fine-structure (the bead-like units 45-65 A in diameter) as the previously described immature fibrils. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 32. Carp SLS at 140 seconds in the time-study. A typical immature SLS segment demonstrating the same ultrastructural detail seen in Figure 31. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 33. Carp SLS at 140 seconds in the time-study. Note the variations in length, width and electron density of the different areas of this segment and that one end is relatively discrete compared with the other. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 34. Carp SLS at 140 seconds in the time-study. Note the development of banding (arrows) at the ends of segments and that at least two cross-striations are evident at one end of a segment. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.


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PLATE IX

Figure 35. Carp SLS at 160 seconds in the time-study. There are large numbers of globular units in the extra-fibrillar spaces as well as associated with the segments. The electron density is quite variable from point to point within the SLS and two cross-bands are evident (arrows) at one end of a segment. An ill-defined cluster of tiny globular elements (A) appears to be organizing in part into a more discrete SLS-type segment. Compare the ultrastructural detail at A with that at B. Negative staining with 2% potassium phosphotungstate, pH 7. X 108,500.

Figure 36. Carp SLS at 180 seconds in the time-study. The structure at A appears to be an elongated fibrillar aggregate of the 45-65 A diameter globular units seen in the segment at B and in the unorganized extra-fibrillar substance, yet it does not have the dimensions of a classically described SLS segment. The aggregate at C has an even more obviously globular fine structure. The structure at D might be interpreted as being two SLS segments aligned side-by-side and partially overlapping; the total length is 450-500 A longer than the average SLS segment in this time-study. However, there is no obvious line of separation between these structures and the length is within the range of variation seen in SLS preparations. The segment at E is also long and irregular but the fine structure is comparable to more typical SLS fibrils in the micrograph. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.


35
A


36

PLATE X

Figure 37. Carp SLS at 220 seconds in the time-study. The bulbous projection on the fibril at A appears to be continuous with the "extra-fibrillar" material at the arrows. Similar projections are present on the fibrils at B, C and D. The bulbous projections on the segment at E in a relatively dense and uniform fibril are quite characteristic of mature SLS and are often seen in classically described SLS and in native-type fibrils. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.

Figure 38. Carp SLS at 260 seconds in the time-study. This fibril is quite narrow at one end and widens significantly at the opposite end where the globular nature of its fine structure and connections between the fibril and the beaded filaments in the extra-fibrillar material become more evident. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 108,500.


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PLATE XI

Figure 39. Carp SLS at 340 seconds (5 2/3 minutes) in the time-study. The SLS segments appear to become quite uniform in length and width after 5 minutes, but reference to Tables I and 2 and Graphs I, 2, 3 and 4 indicate that the ranges of lengths and widths observed are not too different from those at earlier time intervals. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 156,000.

Figure 40. Carp SLS at 420 seconds (7 minutes) in the time-study. Although these fibrils are less obviously globular than the early stages of SLS development, the fine structure still reveals the 45-65 A diameter globules and a bulbous projection is evident at A. The segment appears to be continuous with the unorganized "extra-fibrillar" material at B. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 156,000.

Figure 41. Carp SLS at 500 seconds (8 1/3 minutes) in the time-study. The SLS fibrils have become quite uniform in length and width and the cross-striations, noted early at the ends of some segments, is now consistently present at both ends of all the segments. The banding pattern at the center of the SLS fibrils is also beginning to appear. The globular nature of the extra-fibrillar material is still obvious. The bands and the interbands of these segments are composed of the 45-65 A bead-like units noted throughout the formation of the SLS segments. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 156,000.

Figure 42. Carp SLS at 5 1/3 minutes prepared by the loop method. This specimen is from a time period similar to that in Figure 39 which was prepared by the spray method. The bead-like globularity of the ultrastructure is evident in this figure and in Figure 39. Surveys of hundreds of grid areas and micrographs demonstrated that the resulting SLS segments had the same form and ultrastructure by the two methods (loop and spray techniques of specimen preparation). Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 156,000.


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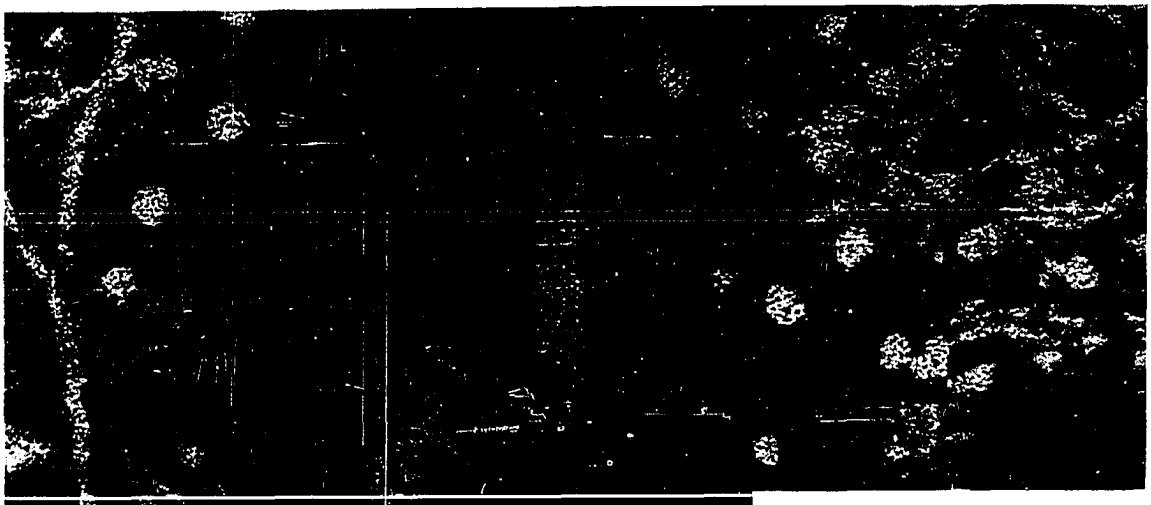

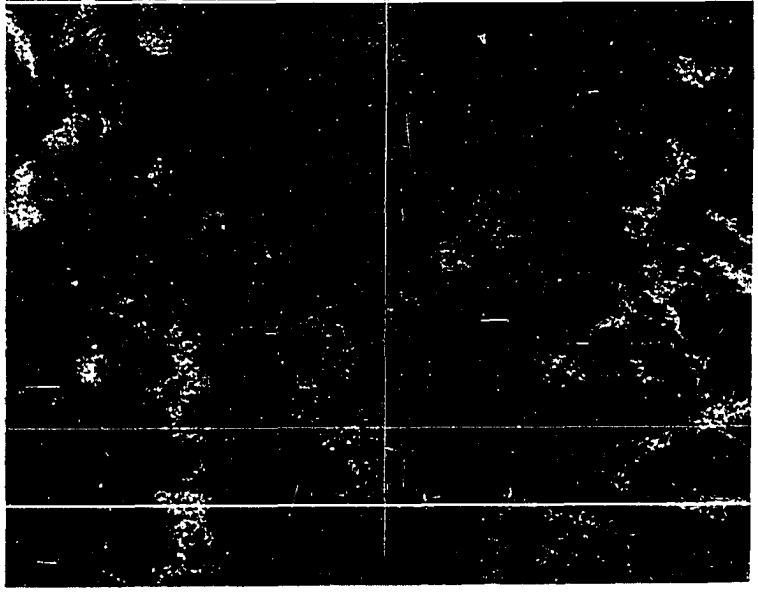

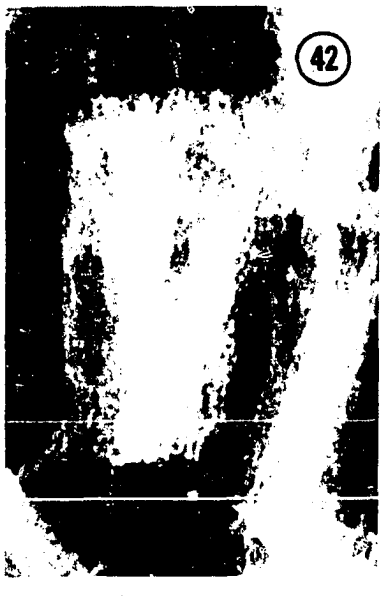
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PLATE XII

Figure 43. Carp SLS at 9 1/3 minutes by the loop method. Several cross-striations are present in each SLS segment. The fibrils are quite electron dense, yet the bead-like globularity of the ultrastructure and the bulbous projections from the borders of the SLS are still evident. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 156,000.

Figure 44. Carp SLS at 9 minutes 38 seconds by the loop method. The globular nature of the fine structure is more apparent than in Figure 43. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 156,000.

Figure 45. Carp SLS at 9 minutes 43 seconds by the loop method. This figure demonstrates a classical SLS morphology and band pattern as revealed by positive-contrast (see Schmitt, Gross and Highberger, 1953 for comparison). Positive staining with 1% w/v aqueous phosphotungstic acid. X 156,000.

Figure 46. Carp SLS at 12 minutes 16 seconds by the loop method. Large globular aggregates are still present in the extra-fibrillar areas and the SLS segments are relatively uniform in size and shape, although the banding is poorly demonstrated here. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 156,000.

Figure 47. Carp SLS at 12 minutes 16 seconds by the loop method. This segment is from a different grid area of the same specimen screen as the one demonstrated in Figure 46; although the contrast is poor, it reveals the banding that is present in these fibrils at this time period. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 156,000.

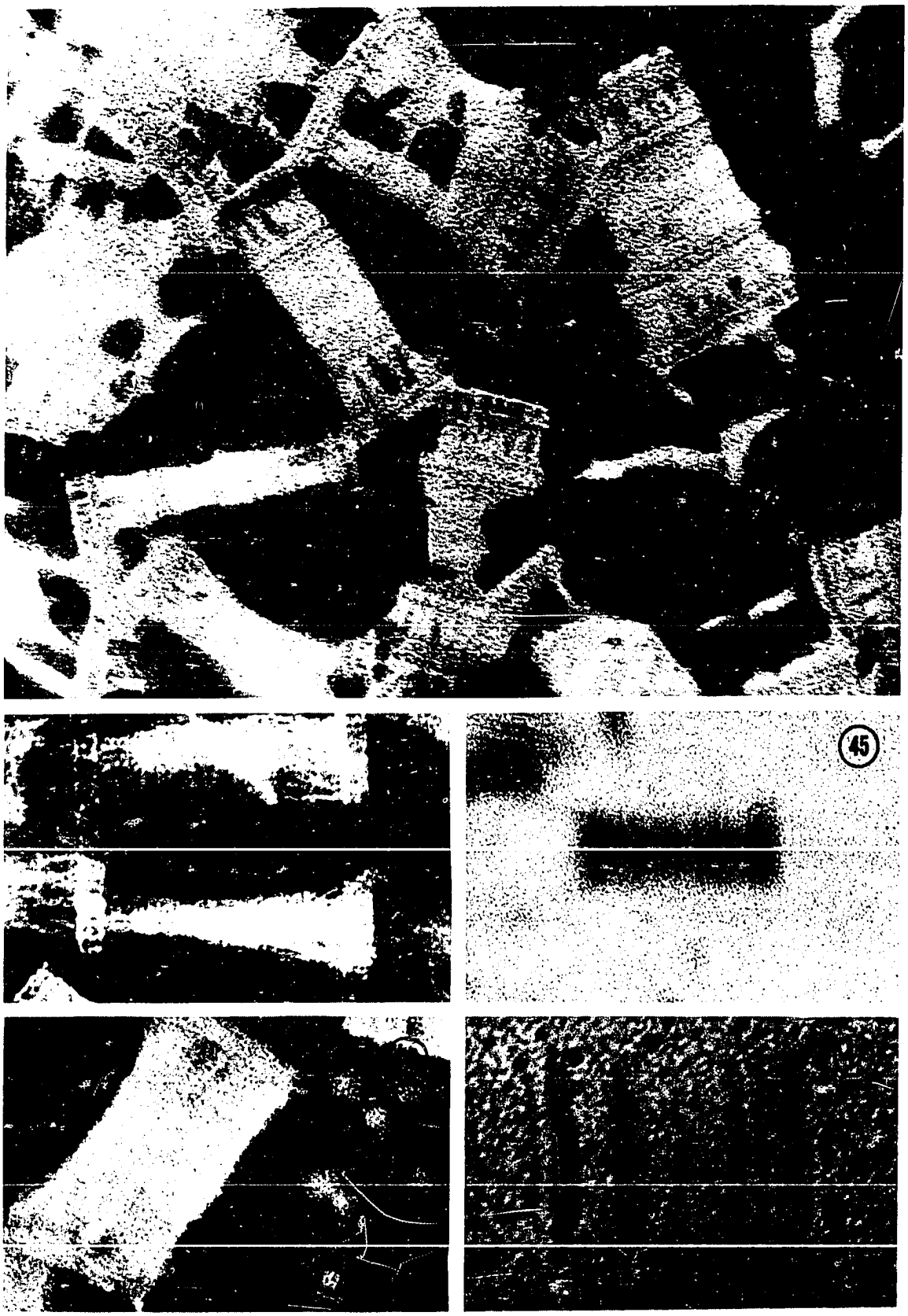
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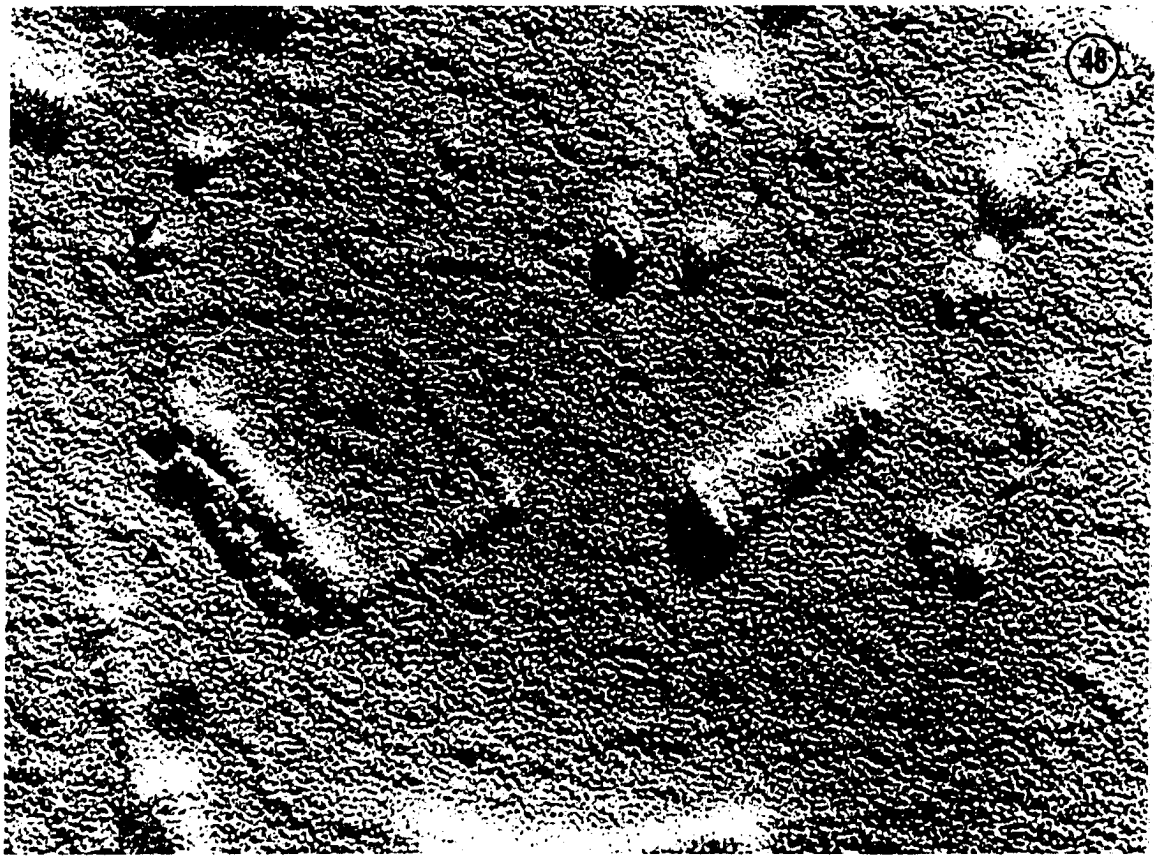
PLATE XIII

Figure 48. Carp SLS at 45 minutes by the loop method. SLS segments prepared by shadow-casting with platinum-palladium (80:20) at a distance of 7 cm and an angle of 10^0 are demonstrated at A. Note also the globular forms (arrows) that are present in this type of preparation as well as in negatively stained material. X 75,000.

Figure 49. Carp SLS at 45 minutes by the loop method. Two SLS fibrils of different widths are demonstrated in apparent end-to-end attachment. The classically described band pattern is evident although the cross-striations are curved, especially at the junction of the two segments. Positive staining with 1% w/v aqueous phosphotungstic acid. X 105,000.

Figure 50. Carp SLS at 9 minutes 43 seconds by the loop method. An SLS segment appears to have formed adjacent to an electron-dense globular mass of unknown composition. Examination of the cross-striations (arrows) reveals them to be "deformed" or altered from their usual nearly perpendicular alignment with the long axis of a segment (compare with segment in Figure 45). The distortion of the band pattern occurs at one end of the SLS and across only one-half its width. Positive staining with 1% w/v aqueous phosphotungstic acid. X 150,000.

Figure 51. Carp SLS at 45 minutes by the loop method. This shadowed specimen demonstrates the cross-striations that are characteristic of mature SLS even in the absence of an electron stain. The arrows point to a distortion of the banding pattern at one end of a segment while the striations at the opposite end remain in their usual relationships. Shadow casting with platinum-palladium (80:20) at an angle of 30^0 and a source-specimen distance of 12 cm. Positive staining with 1% w/v aqueous phosphotungstic acid. X 150,000.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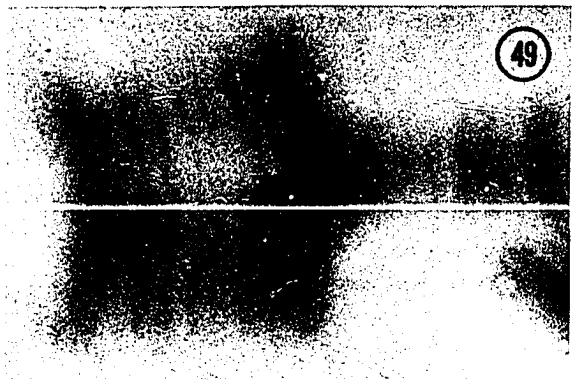
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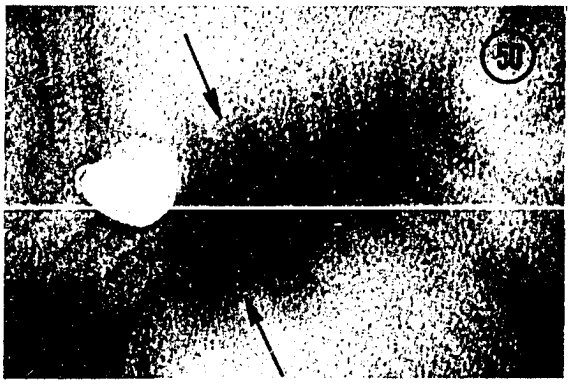
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PLATE XIV

Figure 52. Carp SLS at 75 minutes by the loop method. These SLS now have fully differentiated banding patterns, even by negative staining. The cross-striations at the center are less well-defined than those at the ends of the segments, as has been typical of the development of the SLS fibrils. Segments A, B and C are 1824 A in length at the middle of the fibril. Segment D is 2292 A long. This represents a length difference of about 20% between D and any one of the other three SLS. The 45-65 A globular units noted in the developing SLS are present but are less distinct and obvious and tend to appear in the filamentous structures in the SLS. Larger globular elements can still be seen in the extra-fibrillar substance; these latter appear, in some cases, to be oriented as long strings of globular units (arrows). Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 214,000.

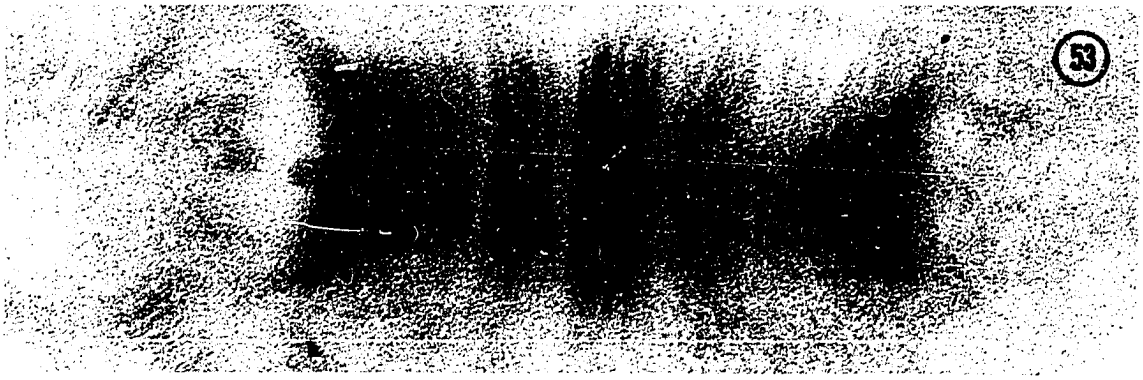

54
C
A

PLATE XV

Figure 53. Carp SLS at 3 1/2 months by the loop method. These appear to be two SLS segments joined along their ends. Although this phenomenon has been noted before in the case of two fibrils of greatly differing sizes (see Figure 49), it has been assumed, in the literature, to represent two separate segments lying end-to-end. There is no evidence to support that assumption and the segments do, in fact, appear to be joined together. Positive staining with 1% w/v aqueous phosphotungstic acid. X 156,000.

Figure 54. Carp SLS at 3 1/2 months by the loop method. This micrograph illustrates the extreme width that some of the older SLS segments achieve and the detailed banding that can be observed. Positive staining with 1% w/v aqueous phosphotungstic acid. X 156,000.

Figure 55. Carp SLS at 3 1/2 months by the loop method. This extremely wide segment is from the same preparation as those in Figures 54 and 55. Negative staining with 2% w/v potassium phosphotungstate, pH 7. X 156,000.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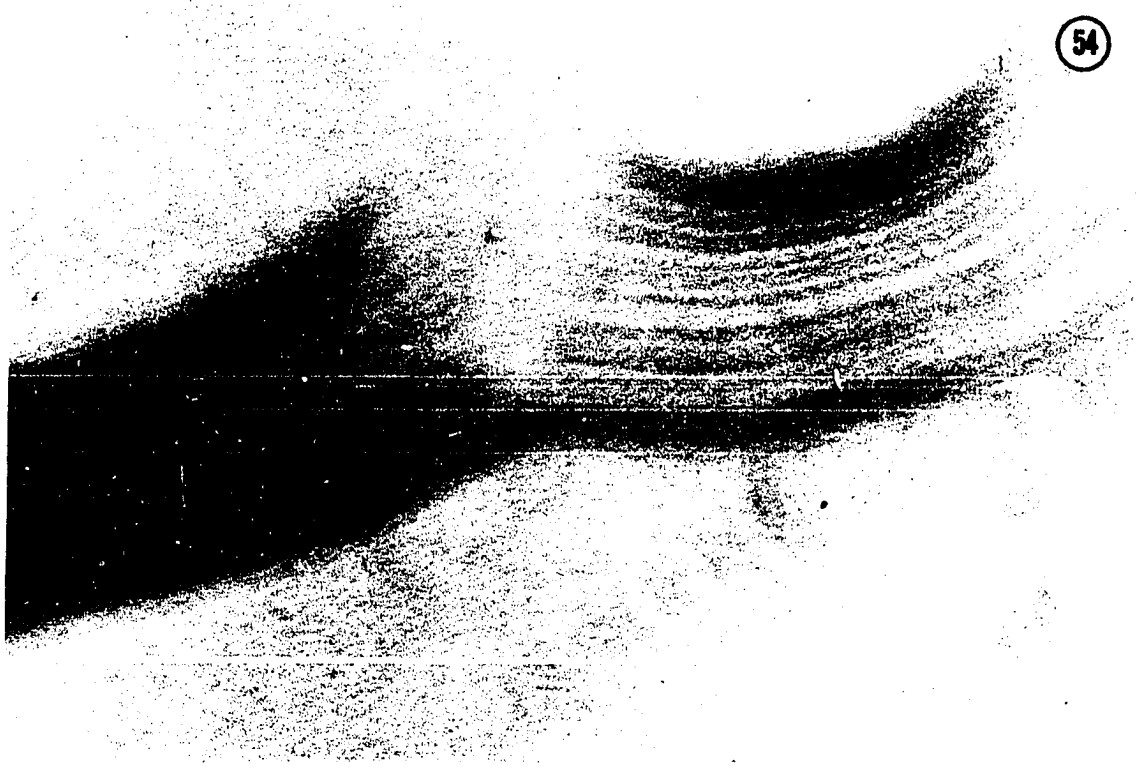
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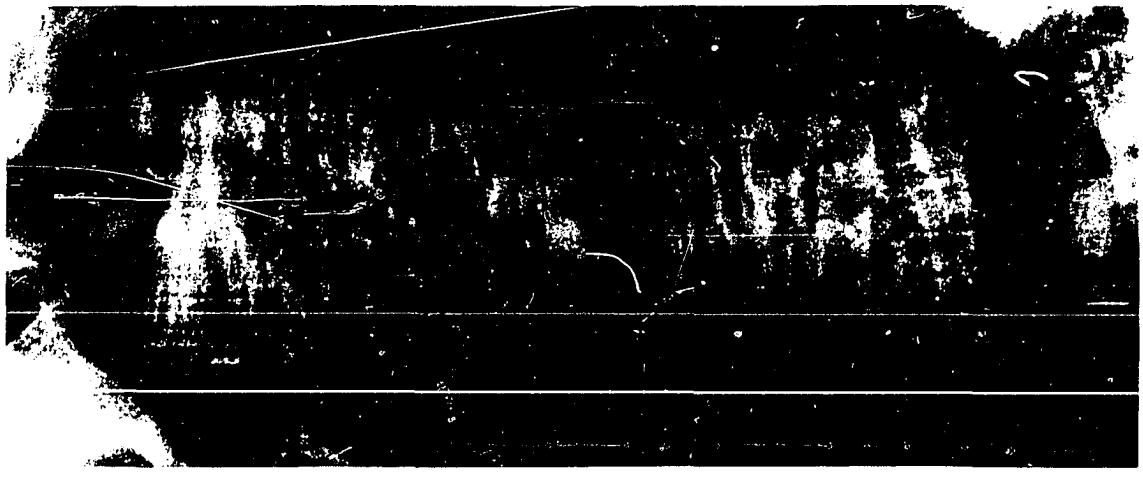

PLATE XVI

Figure 56. Carp SLS embedded and sectioned. The length of this segment is 2894 A near the midpoint of its long axis, a value that correlates with the unsectioned SLS in the time-study. The profile is similar to the unsectioned fibrils also (compare with Figures 51 and 52). The cross-striations that are usually seen in mature, unsectioned, positively-stained SLS are poorly demonstrated in sectioned material (compare with Figure 45). Positive staining with 1% w/v aqueous phosphotungstic acid. X 156,000.

Figure 57. Carp SLS embedded and sectioned. Several structures of various sizes and shapes are evident. Although these were seen as frequently as the longitudinal sections of SLS (Figure 56) in embedded SLS preparations, it is not clear whether these represent cross-sections of SLS or other stages in the aggregation of the collagen molecules. The electron dense structure at A has a thin, neck-like extension that is 257 A across; it appears to be divided lengthwise into two bands of globular elements arranged in a diagonal pattern. The globular units are about 129 A wide and blend into the bulbous portion of the structure. A coiling effect is noted at B. Positive staining with 1% w/v aqueous phosphotungstic acid . X 156,000.

Figure 58. Carp SLS embedded and sectioned. There is a poorly defined banding pattern in the elongated fibrillar segment but the detail is not great. Note also the ovoid structures of low contrast. Positive staining with 1% w/v aqueous phosphotungstic acid. X 156,000.

Figure 59. Carp SLS at 5 minutes 16 seconds by the loop method. The profile of this segment is quite discretely outlined in negative-contrast, especially the ends. The banding that has been demonstrated by other methods is not evident. Negative staining with osmic acid vapors, using a modification of the Barland-Rojkind technique. X 150,000.

Figure 60. Rat tail tendon SLS embedded and sectioned. This micrograph further illustrates the difficulty in obtaining contrast and detail in reconstituted materials that are embedded and sectioned. Positive staining with 1% w/v aqueous phosphotungstic acid. X 146,000.


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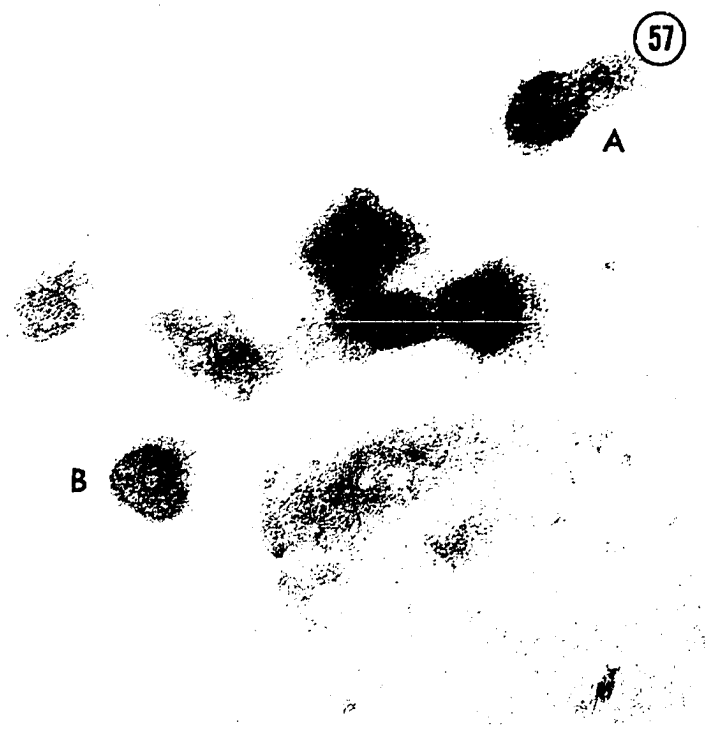
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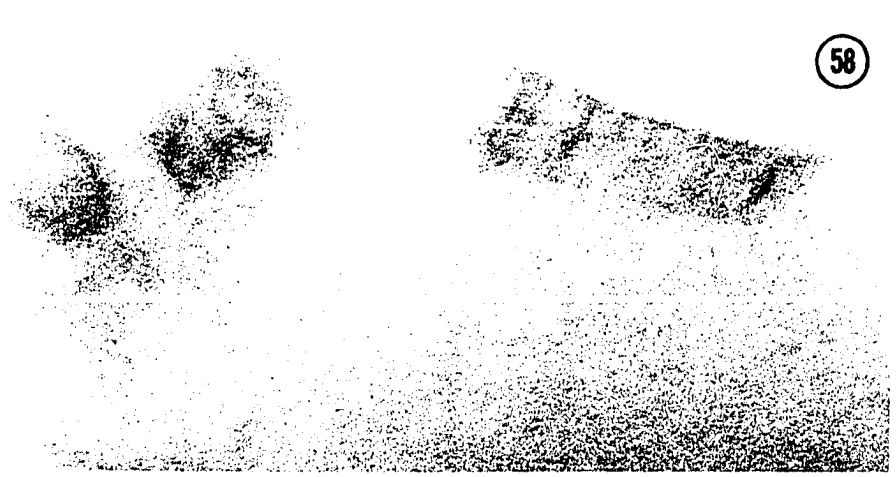
A
B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