THE FINE STRUCTURE AND BIOSYNTHESIS OF SILK FIBROIN

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[*Manuscript received January* 7, 1952]

Summary

Electron microscopic examination of fibrillar fragments produced by the enzymic disintegration of silk fibroin suggests the existence of fine microfibrils about 100 Å in diameter extended parallel to the length of the fibre axis. The microfibrils are similar in width to the crystalline micelles deduced from X-ray diffraction.

Fibroinogen, the soluble precursor of fibrous fibroin extracted from the mature silk glands of the silk-worm, spontaneously separates from dilute solutions in the form of fine microfibrils. This phenomenon appears to be similar to the formation of F-actin from G-actin and fibrin from fibrinogen, and is probably due to an unsymmetrical aggregation of the molecules of the soluble form.

The "soluble silk" of Coleman and Howitt (1947) does not possess the same property of spontaneously fibrillating as does natural fibroinogen. Assuming that a similar phenomenon occurs during the spinning of silk by the silk-worm, a possible course of events during spinning is outlined.

I. INTRODUCTION

Many natural fibres are characterized by an ordered structure extending from the molecular level to the macroscopic and the concept of levels of organization is a valuable aid to their description. A particular interest is the description of the fibre at the level of fine structure since structure at this level may be related to the orderly processes of biosynthesis which brought the fibre into being, and thus provides necessary information for an understanding of these processes. The term "fine structure" is used here in the sense advocated by Picken (1940) to describe structure with a dimension in the range *c.* 50-100 Å and at present most conveniently studied by electron microscopy. A distinction has been made between "spun fibres" and "grown fibres" largely on the basis of their fine structure (Frey-Wyssling 1948). Grown fibres, such as cellulose or keratin, possess a fine structure of microfibrils; whereas this feature is thought to be lacking in a spun fibre such as rayon.

Since there are obvious analogies between the extrusion and drawing of an artificial rayon fibre and the spinning of silk by the silkworm, it is natural to assume that silk is a "spun fibre" in the sense that it possesses little fine structure beyond the micelles indicated by X-ray diffraction. However, this question can only be answered by an actual search for fine structure by electron microscopy or other means. Previous work appeared to show the absence of definite microfibrils (Hegetschweiler 1950; Zahn 1949).

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In this paper two pieces of evidence will be advanced to show that silk fibroin may actually possess a fine structure of microfibrils similar to that possessed by other grown fibres. The first evidence results from an electron microscopic examination of disintegrated silk fibre; the second, less direct, comes from examination of the spontaneous fibrillation of the precursor protein extracted from the mature silk glands of the larvae of the silk-worm, *Bombyx mori* L. The biosynthesis of the silk fibre will be discussed in terms of these findings. Regenerated fibroin made by the method of Coleman and Howitt (1947) has also been examined for fine structure.

II. Experimental

(*a*) *The Existence of Microfibrils in Natural Silk Fibroin*

Silk fibres are too thick to be examined directly by the electron microscope and, as with other insoluble natural fibres such as hair, it is difficult to reduce them to fragments small enough for examination without destroying the evidence of fine structure. It is usually found with hair and the cellulosic fibres that mechanical disintegration produces fibrillar fragments of a great range of widths related rather to the severity of treatment than to fundamental structure; but on the other hand appropriate enzymic disintegration of hair reveals definite macro- and microfibrils and the study of plant cell walls and slimes demonstrates the reality of cellulosic microfibrils (Frey-Wyssling 1950; Preston *et al.* 1948; Wardrop 1949). The first experiments with silk fibres, made with fragments of acid-swollen silk disintegrated in a Waring Blendor, led to a similar mixture of fibrillar fragments of many sizes in the electron microscope. These findings seem to be essentially the same as those reported by Frey-Wyssling (1950), Hegetschweiler (1950), and Zahn (1949).

However, such a result was not unexpected when regard was taken of the consequence of desiccation and the possibility of strong lateral bonding between fibrillar elements, assuming these exist. It was therefore decided, following the experience with wool, to partially digest fibres by means of an enzyme and to examine the residue after mechanical breakdown.

Cocoon silk was reeled off immediately after spinning ceased, chopped into lengths of about 2-3 mm., and washed in several changes of 0.05 per cent. sodium oleate solution at 70°C. to remove the sericin. The de-gummed silk was rinsed in distilled water and then extracted for one day in a Soxhlet extractor using light petroleum b.p. 60-70°C. to remove fatty substances. Purified fibre (2 g.) was then added to 500 ml. of a trypsin solution (a Difco preparation 0.05 per cent., pH 8-8.6) and maintained at 40°C. for 6 months. The trypsin solution was changed once a week over this period. On several occasions the solution became infected with bacteria, which may have assisted the digestion.

At the end of this period the fibre was much damaged, and vigorous treatment in the Waring Blendor produced a suspension of fine fibrillar fragments. By fractional centrifugation the finer material was separated and re-

suspended in distilled water. Drops of this suspension were dried directly on collodion films and lightly shadowed with uranium in the conventional way.

Electron microscopic examination revealed fibrillar material of great diversity of size, much as has been already described. Attention was concentrated on the thinner ribbon-like fragments, in which fine structural details were most likely to be visible. The existence of what seem to be long, thin, parallel microfibrils was evident in micrographs such as Plate 1. It is difficult to measure the widths of fibrils laterally shadowed, but assuming those in Plate 1 are close-packed laterally, a width of not greater than 100 Å can be deduced.

Zahn (1949) has reported fibrils of 90 Å diameter without attributing special significance to them. To judge from his electron micrographs his preparation bears little resemblance to that described here. Hegetschweiler's (1950) preparation is more similar but this author also concluded that no definite microfibrils existed. As mentioned above these observations on silk are similar to those made on cellulose. The earlier electron microscopy of cellulose was carried out on mechanically disintegrated material and fine fibrils of a considerable range of diameter were found. When appropriate material, such as plant cell walls or slimes, was examined, discrete fine fibrils were discovered. It now seems probable that in both materials definite microfibrils are the primary structure and that these may be split longitudinally to yield finer fibrils.

The visual evidence of fine fibrils in silk fibroin provided by electron micrographs, such as that of Plate 1, can be criticized on the grounds that prolonged enzymic digestion may have been responsible for the result. The same criticism could be brought against similar findings concerning hair. Since it is impossible to effect disintegration of these resistant fibres in any other way without obviously damaging the structure, it is possible to meet the criticism only by stressing the apparent individuality of the microfibrils or by finding independent evidence of their existence. Such evidence may be found by new techniques in electron microscopy or by low-angle scattering of X-rays, but is not available at present. Low-angle discrete X-ray reflections indicating lateral spacings of the order of 100 Å have not been observed, but the existence of long, thin, crystalline micelles has been postulated to explain the observed size of X-ray reflections in the wide-angle pattern. The micelle predicted from X-ray work and the microfibril found by electron microscopy are of a similar order of width and may well be the same structure.

Actually the diameter of the microfibrils seems to be rather larger than the breadth of micelles computed from X-ray measurements and they are a great deal longer. However, the apparent differences in size may be merely a result of experimental difficulties. For instance, since not much reliance can be placed on measurements made on micrographs of metal-shadowed detail *c.* 100 Å in width, the diameter of the microfibrils may be somewhat less than 100 Å; on the other hand, X-ray estimates of micellar width are likely to be underestimates, since factors other than micellar size contribute to a broadening of X-ray reflections. Should the diameter of microfibrils prove beyond question to be greater than the micellar breadth, as may be the case if some estimates (Preston *et al.* 1948) of fibrillar width are correct, it will be necessary

to assume that within the microfibrils, which are assumed to be the original structures related directly to synthesis, a secondary crystallization of chains occurs leading to the "fringed micelles" of earlier writers (Meyer 1942).

From the occurrence of flattish sheets of microfibrils one might infer a preferred tendency towards unilateral association, but there was no evidence that these fine fibrils were grouped into definite macrofibrils as in the keratins.

(*b*) *The Spontaneous Formation of Fine Fibrils in Extracts of the Silk Gland of* B. mori

Certain aspects of the formation of silk fibre by the silk-worm are familiar and the analogy to the spinning of artificial fibres is often referred to. The proteins, which are the precursors of the insoluble fibroin and sericin, accumulate in solution in the silk gland as a result of the activity of the sericinogenic cells lining the gland (Bergmann 1939). In this article we are not concerned with the primary synthesis of proteins by these cells although they, with their remarkable nuclei, would repay further study by modern methods. The present problem concerns solely the mechanism whereby the soluble proteins, secreted by the cells, are converted into the insoluble fibre during the spinning of the thread of the cocoon.

The protein concentration in the gland is very high (about 30 per cent. of the fresh weight) and the solution is in a state akin to supersaturation. Mechanical disturbances such as stretching or flattening readily provoke the transformation into the insoluble form. The stretched gland contents yield a good fibre-type X-ray pattern identical with that of silk fibre (Meyer 1942). The untreated dried gland gives a pattern equivalent to that of disoriented fibre (Trogus and Hess 1933). Experiments have been in progress in this laboratory concerning changes in the X-ray pattern occurring during transformation. However, these need not be reported, since similar experiments have been published (Shimizu 1941; Kratky, Schauenstein, and Sekora 1950).

The contents of the gland are too concentrated to provide material suitable for electron microscopy. It was therefore necessary to explore the possibility of using dilute solutions. Various earlier workers, including Foa (1912), Ludwig, and Dubois (see Bergmann 1939), found that the gland contents were partly soluble in water. According to these workers it is the precursor of fibroin (fibroinogen) alone that is soluble in water—the sericin precursor being insoluble. The correctness of this observation was assumed in the present work, but needs confirmation. The aqueous solution of fibroinogen behaves similarly to solutions of other proteins and this justifies the conclusion that insoluble fibrous fibroin is developed from a non-fibrous precursor and that the transformation may bear a resemblance to that of other fibre-forming systems better studied, e.g. G- and F-actin, fibrinogen-fibrin. Unfortunately, there is no information available concerning the molecular characteristics of fibroinogen, but it is hoped to obtain this shortly.

Solutions of fibroinogen freshly prepared have a slight turbidity, which increases in the course of a few hours. After a variable length of time (4-12

hours) the protein separates as a flocculent precipitate. This phenomenon was studied with the electron microscope.

Silk glands were removed from fully grown caterpillars of *B. mori*, washed briefly in distilled water, and placed in distilled water at room temperature. The gland membrane was gently slit to allow the contents to enter solution and the whole stood for about one hour without disturbance. At the end of this time the extract was gently stirred and the undissolved material centrifuged off. The extract was faintly turbid and contained approximately 0.1 per cent. of dissolved protein. The observations previously reported by Foa (1912) were confirmed. Drops of this solution and that of a further solution diluted to 1/10th strength were dried directly on collodion films, shadowed with uranium, and examined in the microscope. Such specimens revealed only indeterminate clumps of material, no fibrous and no well-defined particulate forms.

On standing, the turbidity of the extract of the gland increased and in some hours (9-24) a precipitate began to separate. After centrifugation and washing the precipitate was X-rayed and was found to yield a pattern similar to unoriented silk fibre. Drops of the turbid extract were dried on collodion films and shadowed. Examination in the electron microscope revealed clumps of fibrils such as shown in Plate 1. The turbidity is thus associated with the spontaneous development of these fibrils, which become entangled and separate as a precipitate.

On comparing electron micrographs such as Plates 1 and 2 with micrographs of actin and fibrin, one notes in the silk derivatives the absence of a tendency for the fine fibrils to clump together, e.g. as tactoids, or to build larger fibrils, which is so obvious a characteristic of the other two fibre-forming proteins. Whether this feature is related to the absence of macrofibrils in silk fibre is an interesting question.

The observation that fibroinogen in dilute solution forms microfibrils spontaneously is not direct evidence that naturally formed fibroin possesses a fine structure, since it involves the assumption that the fibrillation in dilute solution is similar to that which occurs in the concentrated solution of the actual gland. The problem needs fuller investigation and the present evidence is only suggestive. A difference in appearance — particularly length — between the microfibrils of silk fibre and these of Plate 2 is obvious. This does not affect the conclusion that fibroinogen possesses the property of forming fibrils by what appears to be a simple aggregation in the absence of mechanical orienting influences, a property displayed by other fibre-forming substances and suspected of being a fundamental step in the formation of biological fibres.

(*c*) *Fine Structure of Regenerated Silk Fibroin*

Coleman and Howitt (1947), whose valuable work redirected interest towards this fibre as a useful substance for the study of fibre formation, prepared soluble derivatives by dispersing the fibre in cupriethylene diamine solution. They called their soluble silk "fibroinogen" and assumed its identity with, or

close similarity to, the soluble precursor of the silk gland, to which this name is more correctly applied. However, the molecular characteristics of their soluble derivatives cannot be ascribed with certainty to the silk gland proteins until a further comparison of the two materials is made. Silk fibre regenerated from the solutions of Coleman and Howitt gives an X-ray pattern similar to the original silk. This also does not suffice to establish identity since a variety of break-down products of sufficiently high molecular weight could be expected to yield the same large-angle pattern as is also the case with solutions of wool (Mercer 1949), feather (Lundgren 1949), or cellulose (Meyer 1942).

Since soluble silk can be readily induced to revert to the insoluble form it was decided to examine such regenerated fibre for the presence of fine structure.

Silk fibroin prepared as in Section II(*a*) was dispersed in 50/50 cuprie-thylene diamine as described by Coleman and Howitt (1947). The solution was dialysed until free from copper, and fibrous products prepared in two ways:

(i) By allowing the solution to stand until a surface scum of insoluble regenerated fibroin appeared.

This was scraped off, washed, and dispersed into fine fragments in the Waring Blendor. Examination in the light microscope and in the electron microscope showed that the fragments formed thin sheets which did not seem to be mats of a fibrillar texture but were more homogeneous.

(ii) By diluting the solution to about 0.5 per cent. protein and allowing it to stand until it became turbid and set to a weak gel. This gel was then dispersed in water and examined with the electron microscope. In this case the fragments were found to be particulate and of no ascertainable structure.

III. Discussion

At the present time great interest is being displayed in the problem of the formation of fibres in biological systems. In most cases it is thought that a non-fibrous percursor is at first synthesized and that this is subsequently transformed into the fibrous form. Silk formation obviously offers a good example of this change. Theories to account for the transformation (often referred to as the G-F transformation (Wyckoff 1949)) may belong to one of two classes: (i) *Aggregation theories* according to which the precursor is a more or less symmetrical particle with the property of aggregating to long strings (or more complex formations) forming the protofibrils of the fibre. (ii) *Chain-unfolding theories* in which the precursor molecule unfolds into a long chain, extends, and crystallizes with other chains to form long micelles of the "fringed" variety (Meyer 1942). In the protein fibres the chain-unfolding hypothesis is often stated to be a type of extreme "denaturation." Under natural conditions, there is no convincing evidence of denaturation, but it may occur in the process of making fibres artificially.

Experimental evidence to distinguish between the implications of the two theories is not easy to come by since, as far as most properties of the fibres are concerned, both theories explain the facts equally well. Instances in natural systems, in which a seemingly slight, even reversible, change precipitates the appearance of fibrils, are not easily explained by denaturation and seem to point rather to aggregation. It is at the level of fine structure, however, where the two theories, interpreted in their simplest forms, predict different structures. The most probable fine structural feature to be expected from the crystallization of long chains is the "fringed micelle"; on the other hand linear aggregation might be expected to produce long, discrete fibrils of constant diameter. The fringed micelle was proposed to reconcile the micellar structure, implied by X-ray studies, with the co-existence of chains longer than the micelles themselves. Only since the development of the electron microscope has the existence of long microfibrils of constant diameter been established and given support to aggregation theories.

On the grounds of its established molecular structure (Meyer 1942) and the way in which the silk-worm spins the fibre, silk fibroin would seem to be a clear example of a fibrous form produced by the unfolding of molecular chains. However, the present work shows that silk, in common with other protein fibres, possesses a fine structure of microfibrils and that moreover fibroinogen, the soluble precursor of the insoluble fibre, displays the property of forming fibres spontaneously without the participation of mechanical forces. These findings emphasize that silk has greater structural similarity to other natural protein fibres than to artificial fibres produced by extrusion and drawing. The activities of the silk-worm during spinning are thus not entirely analogous to those occurring in artificial spinning.

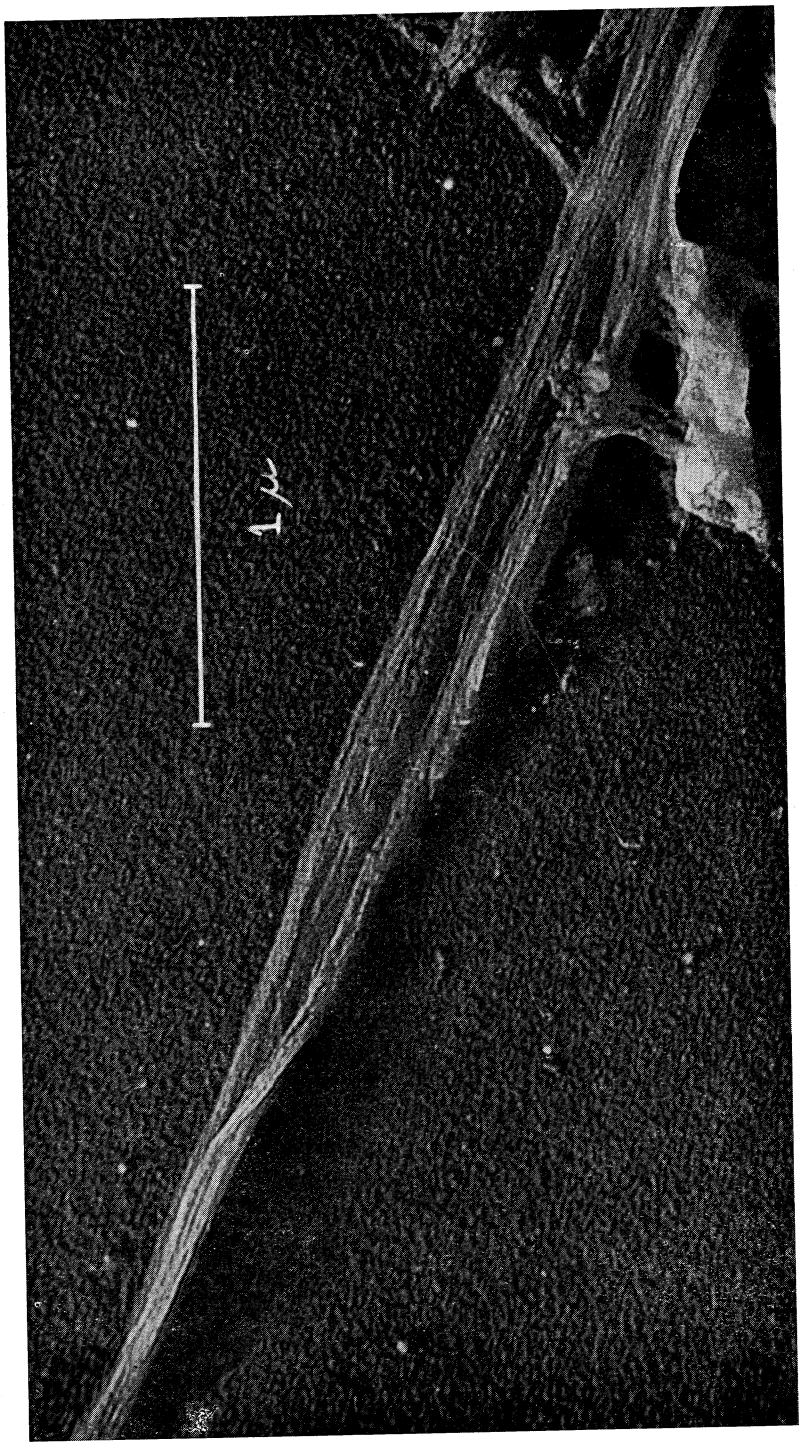
Assuming the existence of microfibrils and their formation by aggregation a possible sequence of events during the natural spinning process would be as follows:

(i) Syntheses of soluble precursor (G-form) — fibroinogen (primary synthesis);

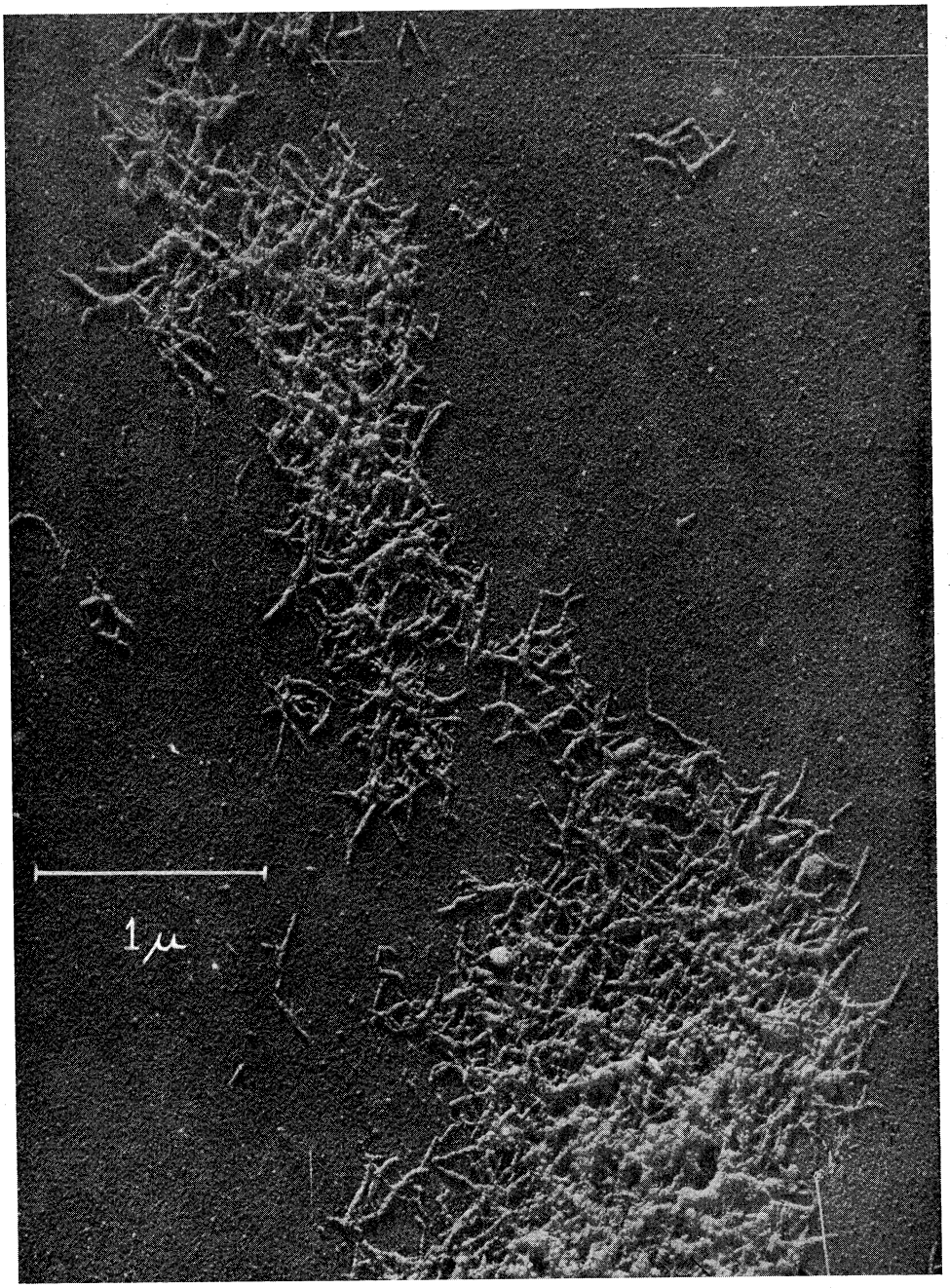
(ii) The aggregation of the precursor to give microfibrils (protofibrils), which may be called *fibrillation*;

(iii) Orientation of the mass of microfibrils by the viscous shear produced during the passage of the gland contents through the silk press and subsequent extension.

The difference between this theory and the accepted scheme lies in the absence of molecular chain unfolding produced by stretching. It is assumed that the precursor molecules are incorporated intact into the protofibrils. Clearly, in order to produce the molecular orientation revealed by X-rays, it must be further assumed that these molecules are oriented on aggregating to form the fibril.



Fibrillar fragment from silk subjected to prolonged digestion with trypsin. Note fine, parallel fibrils. Shadowed with uranium.



Fine fibrils appearing spontaneously in dilute solutions of fibroinogen obtained from the silk gland. Shadowed with uranium.

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