VITROSIN: A MEMBER OF THE COLLAGEN CLASS*, ‡

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Fibrous structural elements in the vitreous body were first demonstrated by darkfield and phase contrast microscopy (1–3). Early electron microscope investigations of fixed vitreous by Schuchardt and Knoch (4) and Schwarz (5–6) showed a network of thin fibrils. Matoltsy, Gross, and Grignolo (7) reported electron microscope studies on the fibrous residues of fragmented whole bovine vitreous body which revealed the presence of three distinctive fibril types. The most abundant form was an indefinitely long, seemingly smooth fibril of the order of 200 A in width. The second less common type was a thin (*ca.* 200 A) beaded fibril with an axial period averaging 610 A. An occasional typical collagen type fibril was also found in the early preparations. Subsequent examination of very carefully dissected specimens failed to reveal the last described fibril, suggesting an extraneous source. Origin of the other two fibril types in the vitreous substance was conclusively demonstrated by obtaining material from single eyes through a trocar introduced through a clean slit in the external membrane of the vitreous body exposed by cutting the sclera equatorially, also *via* an anterior approach after careful removal of the cornea, iris, and lens (Gross, unpublished data).

Recently Matoltsy (8, 9) obtained relatively homogeneous (by electron microscopy) preparations of the "smooth" fibril from pooled bovine vitreous by alternate suspension and reprecipitation with weak alkali and acid. The preparation showed high anomalous viscosity and strong streaming birefringence.

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Synthetic fibers made from stretched precipitates showed positive form and intrinsic birefringence. Chemical analysis revealed 15 per cent N, little or no aromatic- or sulfur-containing amino acids, and 8 per cent non-hexosamine–containing carbohydrate. This protein, named "vitrosin" by Matoltsy (8), constituted about 0.01 per cent of the fresh vitreous body.

That the vitreous contained collagenous material was suspected long ago by Morner (10) and Young (11) on the basis of its gel-forming properties after boiling in water. Pirie (12) studying the whole residual protein found further structural and chemical evidence by x-ray diffraction and paper chromatography for the presence of a collagenous component.

Collagen may be most usefully defined as a class of fibrous proteins exhibiting a characteristic wide-angle x-ray diffraction pattern and in most instances showing evidence of a fiber axis macro period of about 640 A by x-ray diffraction or electron microscopy (13). All members of this class studied to date have revealed characteristically large amounts of hydroxyproline, proline, and glycine and relatively little of the aromatic- and sulfur-containing amino acids (14, 15).

The structural and chemical studies described here definitely identify vitrosin, a component of the residual protein, as a member of the collagen class.

EXPERIMENTAL

Preparation of Material.—Vitrosin was isolated and purified as previously described by Matoltsy (8). Vitreous bodies were carefully removed from 50 fresh cattle eyes, inspected for contamination, washed with water, and fragmented in the cold in a Waring blendor for 5 minutes. The suspension was centrifuged cold at 1800 *g* for 7 minutes and the viscous, water-clear supernatant recentrifuged at 22,000 *g* for 30 minutes. This gave a white, gelled sediment. The crude vitrosin sediment was resuspended in an equal volume of water by moderate shaking for 5 to 10 minutes and recentrifuged at 1800 *g* to remove clumps. The supernatant was diluted with an equal volume of 0.5 per cent KCl with stirring. Vitrosin was precipitated at pH 5–6 by dropwise addition of dilute HCl. The precipitate which adhered to the stirring rod was removed, washed in water, further purified by redispersion in 0.01 N KOH, and reprecipitated by acidification followed by dialysis against H_2O.

X-Ray Diffraction.—Small bits of clotted vitrosin were stretched between forceps and allowed to dry as fibers. These were bound together in bundles 0.5 to 1 mm. in diameter and about 5 to 10 mm. long with a little duco cement at each end, and irradiated with Ni-filtered Cu K_α radiation with the beam passing perpendicular to the fiber axis. For wide-angle patterns, a 5 cm. specimen-to-film distance was used, and small-angle results were obtained with the specimens under vacuum in cameras described by Bolduan and Bear (16) and having *fd* values of 400 and 800 A.

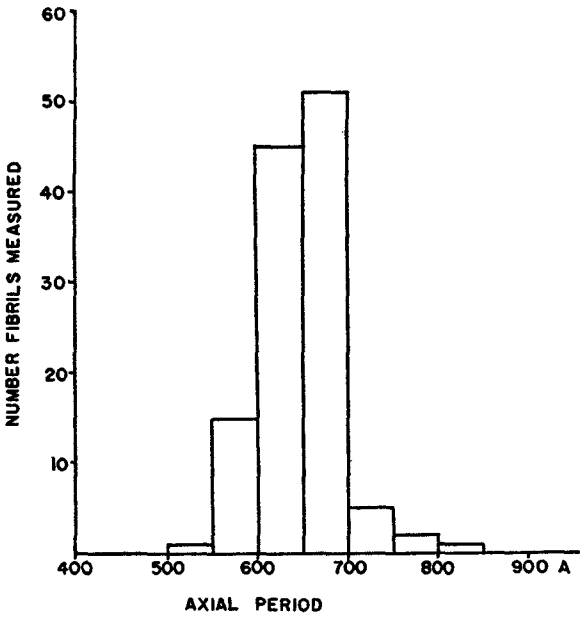
Fig. 1 is a reproduction of a typical wide-angle pattern yielded by these samples. The meridional arc characteristic of collagen measured 2.88 A, the strong equatorial spot 11.7 A, and the more diffuse equatorial diffraction, 4.3 A. The off-meridional arcs corresponding to a spacing of 7 to 8 A belong to a 9.6 A layer line observed more clearly in better oriented collagen samples. The figures cited are the average of measurements from 5 films.

The insert to Fig. 1 is a reproduction of the best small-angle pattern obtained. In general the vitrosin diffracted poorly to small angles. However, orders with indices 3, 5, 6, 10, 11, and

12 (with the 6th outstanding) have been observed for an average spacing of 625 A, which is in a range typical of air-dried collagens. One specimen also yielded a faint equatorial spacing of 93 A, which is unusual.

Electron Microscopy.—Very dilute aqueous suspensions of purified vitrosin were applied to the collodion-covered specimen screens for several minutes, were drained and stained with 1 per cent PTA buffered at pH 2, 5, 4.9, 7.0, and 8.0 in an effort to obtain maximum staining. pH 4.9 proved most satisfactory. Some of these preparations were shadowed lightly with chromium at a 15° angle.

Electron micrographs of PTA-stained preparations revealed a faint but definite axial periodicity (Fig. 2) averaging 640 A (Text-fig. 1) in fibrils measuring about 100 to 150 A in



TEXT-FIG. 1. Frequency distribution curve of axial periodicity observed in PTA-stained vitrosin fibrils. 120 fibrils measured.

width. The broader ones are actually small bundles as can be seen in larger fields. Because of the very low contrast no intraperiod structure could be observed. Shadowed fibrils measured 150 to 300A wide, as reported previously (7). Only fibrils lying parallel to the direction of the shadow were measured. Only in the most favorable instances could the axial period be observed in short regions of fibril bundles in shadowed specimens (Fig. 3). Individual fibrils looked essentially smooth in high resolution, high magnification pictures; apparently the periodic contour variations are neither great enough nor abrupt enough for resolution.

Chemical Analyses.—Two dimensional chromatograms of acid hydrolysates by the method of Redfield (17) were used for qualitative determination of amino acid composition. Solvent systems used were methanol-water-pyridine and *n*-propanol-water-diethylamine. 6 N HCl hydrolysates were desalted on a 5 cm. dowex 50 column. Determinations of hydroxyproline by the Martin and Axelrod (18) modification of the method of Neuman and Logan (19) and glycine by the Christensen, Riggs, and Ray (20) modification of the procedure of Alexander

et al. (21) were made on the same 6 N HCl hydrolysate of 2 to 5 mg. of vitrosin. Determinations on standard gelatin hydrolysates were run simultaneously as well as the usual amino acid standards.

The amino acid pattern is essentially the same as that of cowhide gelatin, in both type and order of magnitude. The proline and hydroxyproline spots are about equal in intensity.

Hydroxyproline content was 11.7 per cent and glycine 19 per cent as compared with 14.4 and 28 per cent for cowhide gelatins. Vitrosin differs from most other vertebrate collagens (14) in its relatively low glycine/hydroxyproline ratio, 1.65 as compared with 2.0. In this respect it is similar to the collagen fibers of the marine sponge (22).

DISCUSSION

Since hyaluronic acid is a prominent constituent of the vitreous humor and since it and collagen are characteristic mesenchymal components, it is not surprising to find collagen present.

The wide-angle x-ray diagram and the macro period at small angles indicate that vitrosin is a member of the collagen class of proteins. However, the intensity distribution at small angles is atypical for dry vertebrate collagens and is in some respects similar to that of invertebrate collagens, particularly in lacking a 9th order (23).

The 93 A equatorial spacing is probably a rough measure of fibrillar widths, which, according to electron optical studies of stained fibrils, are about this order of size. Other collagens have much thicker fibrils, and corresponding diffractions measuring their transverse packing would be unresolved from the central undiffracted x-ray beam. The average fibril width measured in electron micrographs of shadowed preparations is more than twice that given by the equatorial spacing in the small-angle x-ray diagrams and by measurements of pictures of unshadowed fibrils. The discrepancy is probably a result of the flattening of the fibril on the specimen screen and the piling up of metal on the shadowed preparation.

Since the axial periodicity as visualized in electron micrographs is discernible chiefly in fibril bundles and rarely visible in individual fibrils one may argue that the substance giving rise to the periodicity is extrafibrillar and in some way superimposes the periodicity on the fibrils. This argument cannot be completely ruled out at this time. However, the wide-angle diffraction reveals no significant amount of oriented material other than collagen. If an amorphous material did, indeed, combine with the fibrils to produce a periodicity of 640 A this would still represent a major contribution of the fibrillar component, as pointed out by Gross, Highberger, and Schmitt (24).

If 8 per cent carbohydrate (previously determined by Matoltsy (8)) is subtracted from the protein the hydroxyproline value is raised to 12.6 per cent, that found in purified steer corium. However, if the hydroxyproline content is calculated with the assumption that 15 per cent N measured earlier (8) really represents the nitrogen content of vitrosin plus non-protein constituents and

that the vitrosin contains 18 per cent N as does hide collagen, then the hydroxyproline content would be 13.9 per cent. It is evident that the measured carbohydrate moiety does not completely account for the low nitrogen.

Young and Williams (25) reported 15.4 per cent hydroxyproline in a vitreous fraction containing 13.0 per cent N. This is higher than that for vitrosin or any member of the collagen class yet studied. The presence of more than 1 per cent of tryptophane and of phenylalanine strongly suggests contamination by noncollagenous protein, which makes the high hydroxyproline even more difficult to explain.

SUMMARY

Vitrosin, a fibrous protein obtained from the vitreous humor of the eye in the form of an indefinitely long fibril about 100 to 150 A in diameter, has been identified as a member of the collagen class of proteins. It is characterized by the collagen wide-angle x-ray diffraction pattern, and axial periodicity of about 640 A determined by electron microscopy and small-angle x-ray diffraction, an amino acid pattern characteristic of collagen as determined by paper chromatography, and a hydroxyproline and glycine content also typical of collagen. The glycine-hydroxyproline ratio is somewhat lower than that for most vertebrate collagens.

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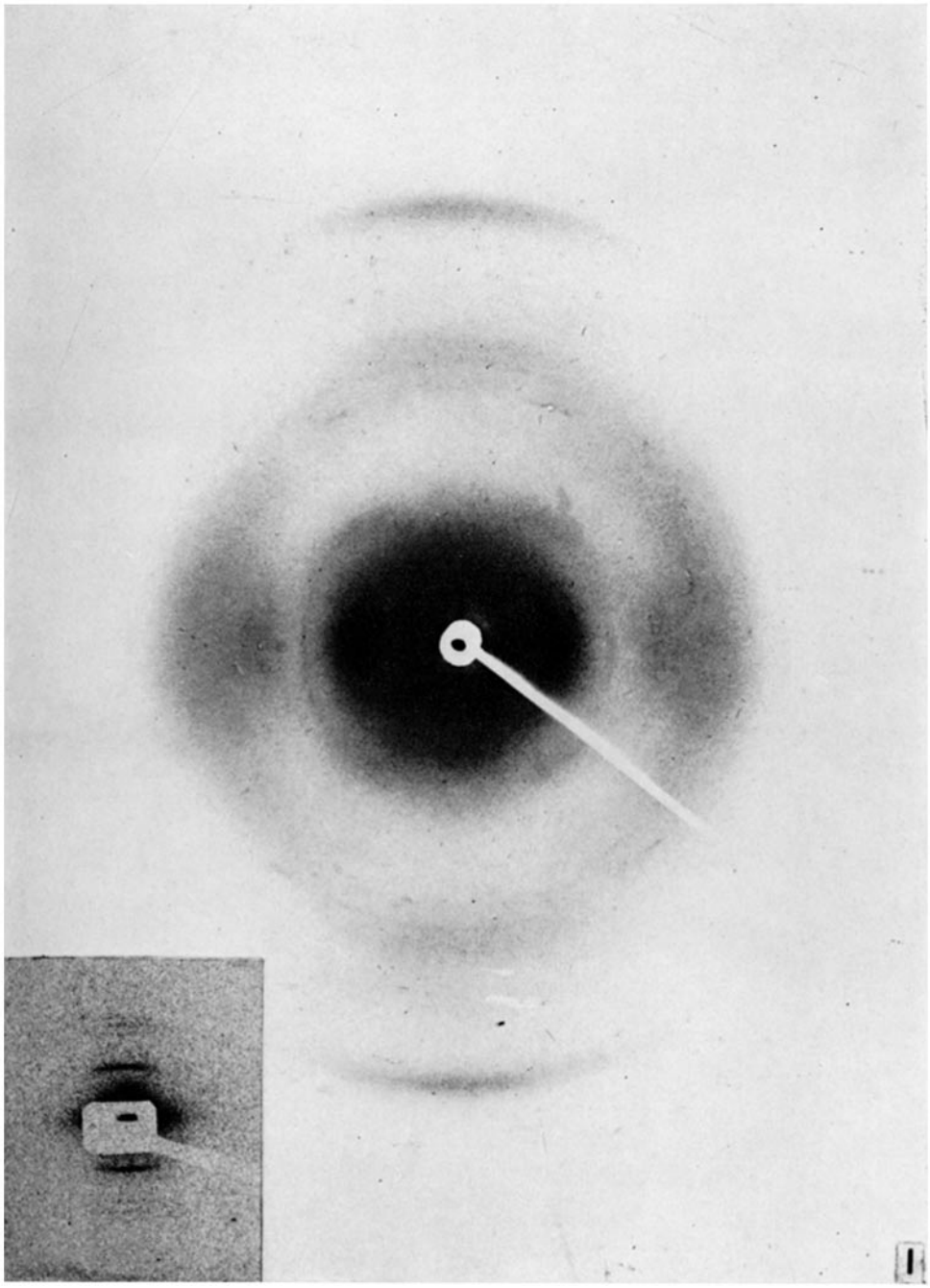
EXPLANATION OF PLATES

PLATE 61

FIG. 1. Wide-angle x-ray diffraction pattern of vitrosin. Insert shows small-angle pattern.

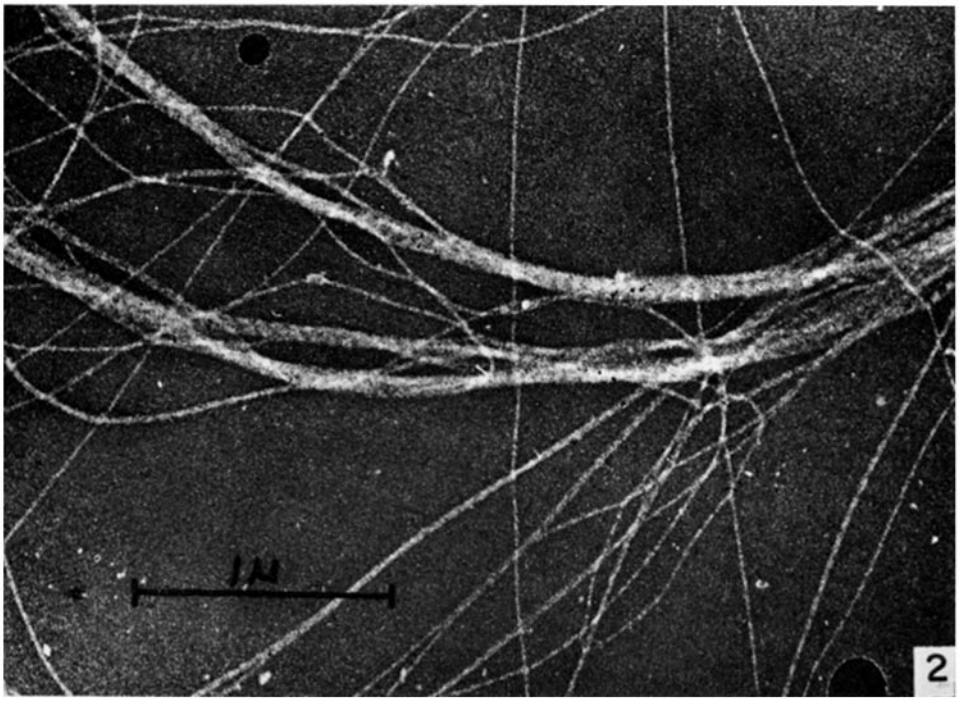


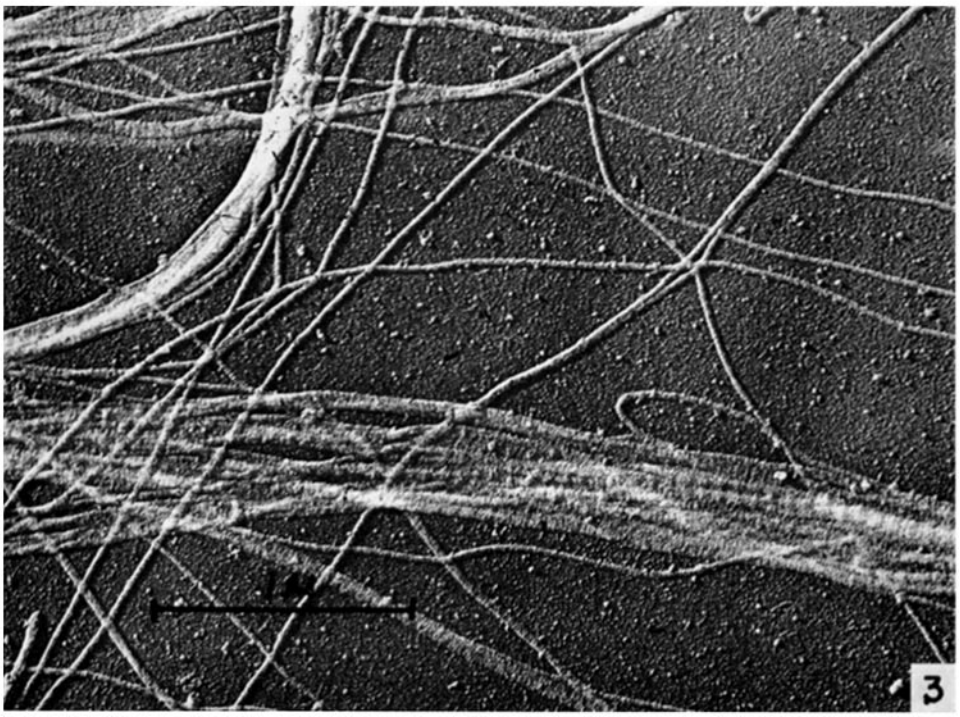
(Gross *et al.*: Vitrosin)

PLATE 62

FIG. 2. Electron micrograph of PTA-stained vitrosin fibrils. Axial periodicity is faintly visible in fibril bundles. × 33,200.

FIG. 3. Electron micrograph of chromium-shadowed fibrils. Period not seen in individual fibrils but just visible in fibril bundles. × 34,200.





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