# A STUDY ON THE STRUCTURAL PROTEIN OF THE VITREOUS BODY (VITROSIN)*

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## INTRODUCTION

The fibrous structural elements of the vitreous body are laid down in the form of a meshwork embedded in a viscous fluid (3, 11). The meshwork was found to be built of microscopic (14) and submicroscopic fibrils (1, 3, 13). The microscopic fibers were first observed in the phase microscope (14) in the fresh tissue; they are invisible in an ordinary microscope because the refractive index of the fibers does not differ much from that of the imbibition fluid. The submicroscopic fibrils were detected in the ultramicroscope by Baurmann and Thiessen (1) who measured fibrils of 300,000 A in length and 150 A in width. Fibrils in this range of dimension were also observed in the electron microscope by Schuchardt and Knoch (13) in formalin-fixed vitreous fragments.

In a previous study it was observed that fibrous structural elements could be sedimented by high speed centrifugation from fragmented vitreous body (7). The most abundant element of the high speed sediment was seen in the electron microscope as a new type of fibril which averaged 250 A in width and exhibited only a suggestion of a very fine axial repeating period. In the present study the nature of this particular fibrous substance was investigated. It was found that it is an insoluble structural protein. Vitrosin is proposed as the name of the protein.

This paper describes the method of isolation and purification of vitrosin and gives details about its physicochemical and chemical properties.

## I

## *Isolation of Vitrosin*

*1. Dissection and Cleaning of the Vitreous Body.*—Cattle eyes were obtained 2 to 4 hours after the death of the animals. In a single experiment 50 eyes were

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used. The experimental material was dissected in the following way. A complete equatorial cut was made through the sclera, choroid, and retina. The vitreous body was allowed to fall away from the retina, while remaining attached to the anterior part of the eye. The vitreous was cut with scissors about 4 mm. behind the posterior lens capsule and dropped into a Petri dish. The anterior part of the vitreous was discarded. The experimental material was carefully examined and all detectable tissue fragments were cut out with the surrounding vitreous tissue. It was then placed in another dish and vigorously rinsed in a large quantity of distilled water for 20 to 30 seconds. The clean material was collected in a beaker packed in ice.

It is important to use separate instruments for each kind of manipulation and to rinse them in distilled water several times. These precautions should be observed because small tissue fragments always adhere to the instrument and to the vitreous and become a possible source of considerable impurity in the isolated substance.

*2. Blending and Centrifugations.*—The experimental material from 50 eyes was fragmented in the Waring blendor for exactly 5 minutes between 0–5°C. The blended tissue formed a foamy fluid. The foam was removed after standing in the refrigerator for 30 to 40 minutes, when most of it spontaneously settled; the rest was removed under negative pressure. This fluid appeared as a viscous, slightly yellowish, transparent fluid.

The centrifugations were done in the International refrigerated centrifuge model PR-1. The first centrifugation was in 100 ml. celluloid tubes for 7 minutes at a temperature between 0–5°C. and at 1800 *G*. A small amount of a brownish sediment was obtained which was discarded. The supernatant was water-clear and showed streaming double refraction, indicating that anisotropic particles were suspended in this fluid.

It was centrifuged in 25 ml. celluloid tubes using the high speed attachment of the centrifuge. Centrifugation was carried out for 30 minutes at a temperature between 15–18°C. and at 22,000 *G*. A white jelly-like sediment was obtained which contained vitrosin. The dried sediment of the processed 50 vitreous bodies weighed 15 to 20 mg.

## II

## *Purification of Vitrosin*

The sticky high speed sediment adhered to the bottom of the centrifuge tubes and did not dissolve or disperse spontaneously in distilled water, but it formed a very fine suspension upon moderate shaking. This property of the substance was used in the purification of the protein.

25 ml. of distilled water was added to each tube which contained the sediment of 25 ml. of vitreous fluid. The tubes were shaken for 5 to 10 minutes. (If shaking is too vigorous or the material prepared from stored eyes, the dispersed substance is readily altered and forms non-dispersible large precipitates.) About 500 ml. of a suspension obtained in this way was centrifuged at 1800 *G* for 5 minutes to remove

large and undispersed particles. The supernatant was diluted to twice its volume with 0.5 per cent solution of potassium chloride and moderately stirred for a few hours, when the soluble substances went into solution. Vitrosin was precipitated from this fluid which was adjusted to pH 5–6 with hydrochloric acid. The acid was added drop by drop and the fluid was constantly stirred with a glass rod. Vitrosin precipitated at once in the form of white, large, sticky, fibrous precipitates which attached to the glass rod and were removed with this from the fluid.

The precipitate was washed in large quantities of distilled water and finally dialyzed in the refrigerator to remove salts. For further purification it was dispersed in 50 ml. of 0.01 N solution of potassium hydroxide. This suspension was diluted ten times with distilled water and stirred for 1 hour. The vitrosin was reprecipitated in the same manner described above.

In most of the experiments twice precipitated substance was used. For the analyses the precipitate was first dialyzed against distilled water for 2 days and subsequently extracted with acetone, ethanol, and ether. Finally it was dried at 110°C. to constant weight. From 50 eyes 10 to 15 mg. of purified vitrosin was obtained, indicating that some of the isolated substance was lost during the purification procedure.

## III

## *Physicochemical Properties of Vitrosin*

*1. General Remarks.*—The fresh precipitate of vitrosin appears as a white fibrous substance. When stretched, it shows considerable elasticity and also double refraction in a polarizing microscope. The precipitate easily disperses in alkaline solutions and forms slightly turbid, very fine suspensions. In this study 0.01 N potassium hydroxide solution was preferentially used as a dispersion medium.

*2. The Form of Vitrosin Particles.*—If a drop of vitrosin suspension is examined in the darkfield microscope, freely moving small particles may be seen, which intensively scatter light and show Brownian movement. A 0.1 per cent suspension shows intense positive streaming double refraction, an observation which indicates that the particles are highly asymmetric.

Electron microscopic examination of the suspensions revealed a large number of thin fibrous elements. The electron microscopy was done by Dr. Jerome Gross in the Department of Medicine, Massachusetts General Hospital. An RCA type EMU electron microscope was used. Once precipitated, the suspension was diluted with distilled water to reduce the amount of material deposited on the collodion-supporting film. The preparations were shadowed with chromium. Electron micrographs show that the preparation is composed of one type of fibrils (Fig. 1). They appear uniform in width and average 250 A. The fibrils either seem smooth or exhibit a suggestion of a very fine cross-striation. The length of the fibrils could not be determined.

*3. The Interrelationship of Vitrosin Particles.*—Suspensions of vitrosin remain non-viscous in concentrations up to 0.02 per cent. Between 0.02 and 0.1 per

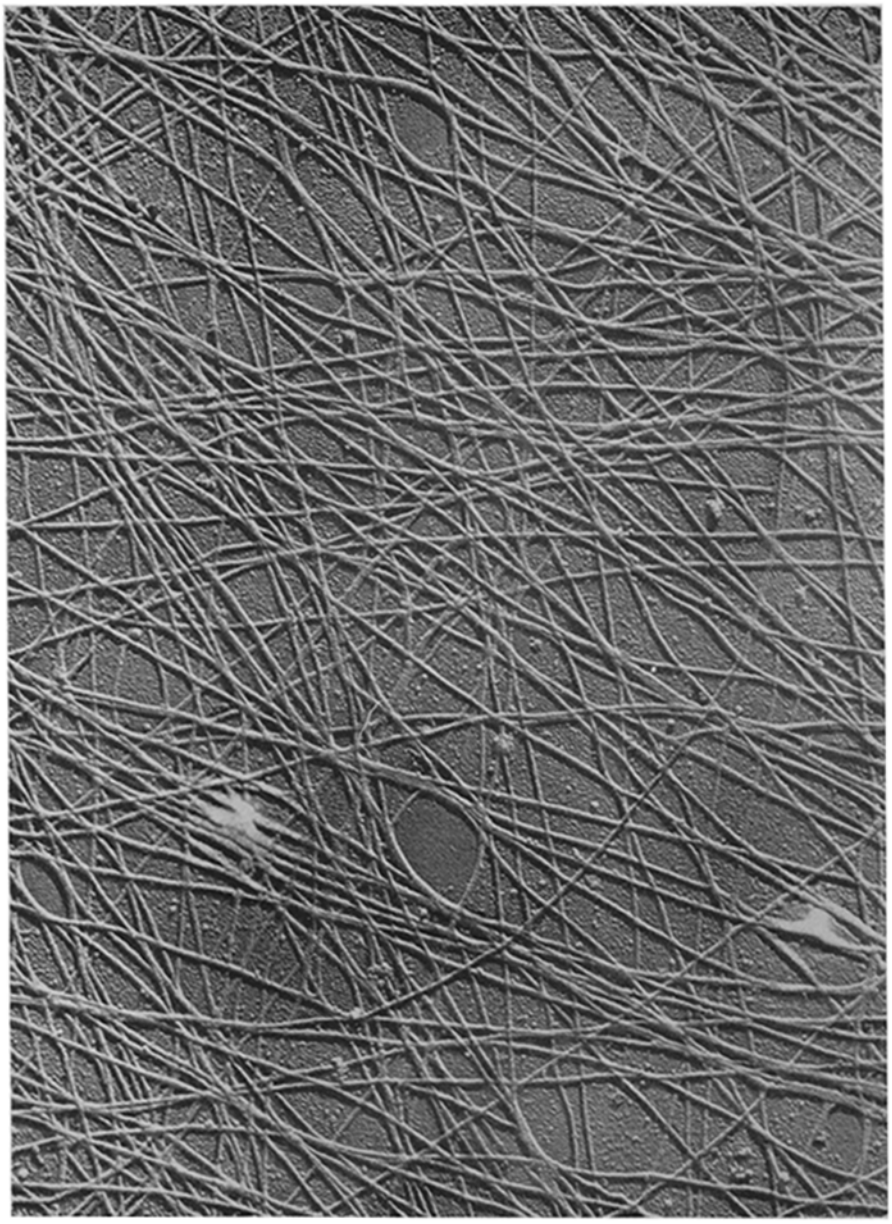

FIG. 1. Electron micrograph of vitrosin. Chromium-shadowed. × 31,000.

cent concentration the suspensions show increasing viscosity. A 0.5 per cent suspension exhibits thixotropy and shows permanent double refraction. A

1.0 per cent suspension forms a rigid gel upon standing for a few days in the refrigerator.

In viscosimetric studies more information was obtained on the interaction of the particles. The Oswald viscosimeter was used and 5 ml. of suspension was investigated at 22°C. The relative viscosity was measured as the function of concentration. The relative viscosity of a 0.02 per cent suspension was 1.1. The viscosity gradually increased with concentration to a value of relative viscosity 1.2 at a concentration of 0.04 per cent. From this point on the viscosity was anomalous and suddenly increased. The relative viscosity of a 0.07 per cent suspension measured 2.6. Above this concentration further measure-

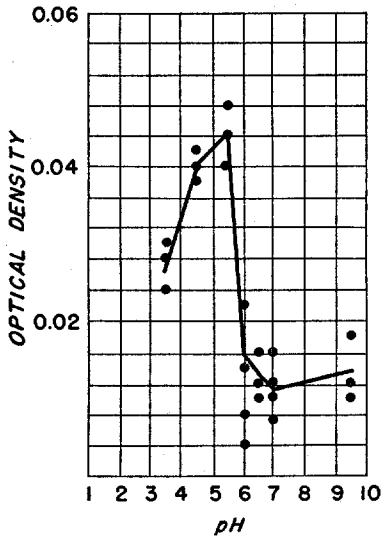


FIG. 2. Optical density of vitrosin suspensions at different pH.

ments could not be performed because the suspensions showed thixotropy. The anomalous viscosity is in all probability due to the disordered arrangement of the long particles which interact with each other. The cohesive forces which stabilize such disordered structures cannot be very strong since the 2.6 value of relative viscosity of a 0.07 per cent suspension decreased to 1.3 if as low a pressure as 8 mm. of water was applied in the viscosimeter.

*4. The Isoelectric Point of Vitrosin.*—The isoelectric point was determined using series of suspensions adjusted to different pH values and the point of optimal flocculation was taken as the isoelectric point of vitrosin. 2 ml. of 0.07 per cent suspension of vitrosin was pipetted into a series of test tubes and gradually increasing amounts of hydrochloric acid were added and the final volume made up to 5 ml. with distilled water. The pH of each suspension was measured with the Beckman pH meter. The optimal flocculations appeared

between pH 4.3 and 6.3. The optical density of the suspensions was measured in the Klett-Summerson photometer using filter 54. The measured values were plotted against pH and the curve obtained showed a maximum at pH 5.5 (Fig. 2). This indicated that the isoelectric point of vitrosin lies in this range. It is understood that such measurements give only approximate isoelectric values. The results are considered preliminary until more accurate measurements can be made with other methods.

*5. The Solubility of Vitrosin.*—The solubility of vitrosin was investigated by dispersing it in different solvents, filtering through a sintered glass filter, and testing the filtrate for protein. Vitrosin was found insoluble in weak or concentrated salt solutions such as sodium chloride, sodium sulfate, sodium phosphate, sodium pyrophosphate, and sodium citrate. It did not dissolve in 0.01 N solution of hydrochloric acid or potassium hydroxide, in 30 per cent urea, and was not digested by trypsin. Vitrosin dissolved within a few hours in hot 0.01 N solutions of hydrochloric acid or potassium hydroxide or in boiling water.

*6. The Effect of Temperature on Vitrosin.*—When a 0.056 per cent suspension of vitrosin was heated, it was observed that the slightly turbid fluid gradually cleared with increasing temperature, the streaming double refraction diminished, and the viscosity decreased. The relative viscosity of the suspension was 1.7 at 20°C. When it was immersed for 10 minutes in a water bath at 40°C. and then cooled to 20°C., the relative viscosity was 1.6. When it was heated to 50°C. in the same way and cooled to 20°C., the relative viscosity decreased to 1.2. The suspension was non-viscous after heating it to 60°C. These observations indicate that the highly asymmetric particles undergo some irreversible modification under the effect of temperature.

When a gel of vitrosin (1 per cent) is heated and the temperature is 60°C., the gel suddenly squeezes out the imbibition fluid and shrinks to a small volume.

Synthetic fibers were prepared from the fresh precipitate of vitrosin by stretching and drying it at room temperature. The fibers showed strong positive double refraction indicating that the particles are oriented parallel to the longitudinal axis of the fiber. When such fibers were immersed in distilled water at 60°C., they shortened 40 to 50 per cent in length.

The observed shrinkage of the gel and synthetic fibers indicates that the shrinkage temperature of vitrosin is about 60°C.

## IV

## *The Composition of Vitrosin*

Information on the composition of vitrosin was obtained by total nitrogen and carbohydrate determinations. The presence of possible phosphorous radicals was determined by phosphorous tests and its relation to hyaluronic acid was determined by hexosamine estimation.

Total nitrogen was determined by Pregl's modification of Kjeldahl's method,

total phosphorous by the Fiske and SubbaRow method (4), total carbohydrate according to Sörensen and Hangraad (15), and hexosamine according to Nilsson's method (10). The analyses gave the following results:

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|---|---|
| Total nitrogen: 14.90 to 15.05 per cent | Total carbohydrate: 7.0 to 9.0 per cent |
| Total phosphorous: none | Hexosamine: none |

The analyses suggest that vitrosin is composed of a protein-carbohydrate complex. Hyaluronic acid seems to be absent from the complex since its basic component hexosamine could not be detected in the preparation; phosphorous-

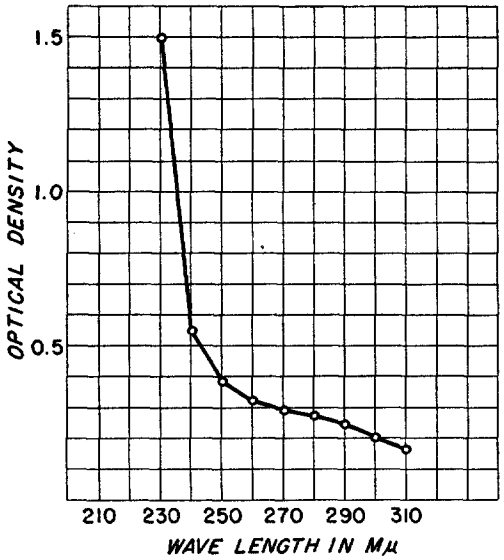


FIG. 3. Absorption spectra of a 0.01 per cent suspension of vitrosin in ultraviolet light.

containing radicals are also absent. The nitrogen content of the complex is low (14.9 to 15.0 per cent) compared to the usual N content of proteins (16 per cent); however, if it is assumed that the estimated N represents the total N of only the protein component and it is recalculated on the basis of carbohydrate-free material, it shows a value between 16.01 to 16.50 per cent which is satisfactory. The nature of the carbohydrate component was not investigated further. The protein component was characterized by amino acid determinations.

Cystine was determined by Block and Bolling's use of the Winterstein-Folin reaction (2). It showed that vitrosin contains 0.5 per cent cystine. The aromatic amino acids were investigated by the spectroscopic method. The absorption spectra of a 0.01 per cent suspension of vitrosin were measured between the

wave lengths 230 to 310 mμ in the Beckman spectrophotometer. The measured absorption curve (Fig. 3) is flat and does not show a characteristic peak to indicate aromatic amino acids (phenylalanine, 258 mμ; tyrosine, 275 mμ tryptophane, 278 mμ). The absorption spectrum of vitrosin is similar to that of purified collagens. Loofbourow, Gould, and Sizer (6) showed that purified collagens characteristically show no absorption in the region from 257 to 287 mμ wave lengths, which was explained by the absence of aromatic amino acids.

These analyses show that cystine and the aromatic amino acids appear in very low concentration or are absent in the protein component of vitrosin. The same amino acids are also very low (cystine 0.1 per cent) or absent (aromatic amino acids) in collagens, which suggests similarity in composition of these proteins.

## V

## DISCUSSION

The fibrous structural elements of the vitreous body were isolated first by Mörner (9) as far back as 1894. He put the vitreous body on a filter paper, and when the fluid part passed through, a fibrous residue remained on the filter paper. Such preparations were studied by many investigators (3, 5, 8, 9, 11). It was agreed that the residue contains an insoluble protein, called residual protein, but different conclusions were drawn about the nature of this protein. Mörner (9) considered it collagen, Duke-Elder (3) described it as allied to mucoproteins, and Mayer (8) classified it as an insoluble mucoid of unknown composition. The different opinions might originate from the complex nature (5, 11) of the residue, which was purified by different methods.

Pirie, Schmidt, and Waters (11) solubilized the residue in hot hydrochloric acid. A fibrous protein was then precipitated by ammonium sulfate. This fraction of the residue was identified by nitrogen, carbohydrate, and quantitative amino acid analyses. Amino acids such as glycine, proline, hydroxyproline, alanine, aspartate, and glutamate were found roughly in the same proportion as in hydrolyzed collagen or gelatin.

Pirie's protein seems to be similar to vitrosin. Its nitrogen (14.8 per cent) and carbohydrate (6 per cent) content is somewhat lower than that of vitrosin. This might be due to the hydrolytic destruction of the protein.

In the present study vitrosin appears as an insoluble native fibrous protein. Although its physicochemical and chemical properties are similar to those of collagen, there is a significant difference in the fine structure of these proteins. Collagen shows a distinct axial repeating period of 640 A (12). This structure is absent in vitrosin.

Vitrosin represents one of the structural elements of the vitreous body. There is no information at this time as to whether vitrosin is uniformly dis-

tributed throughout the vitreous body or is localized in specific regions or structures. Its relation to the other fibrous elements (7) is not yet known.

SUMMARY

A method is described for the isolation of a structural protein of the vitreous body, which has been named vitrosin. The analyses show that vitrosin is a viscous, thixotropic, fibrous protein. Electron micrographs reveal that vitrosin particles are long fibrils, averaging 250 A in width. The isoelectric point was found to be around pH 5.5 and the shrinkage temperature 60°C. Vitrosin is composed of a protein-carbohydrate complex. It contains cystine and the aromatic amino acids in low quantities. Hexosamine could not be detected in the complex.

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