COLLAGEN OF THE EARTHWORMS

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INTRODUCTION

The problem of the complex configuration of the collagen in the Oligochaeta has not yet been conclusively solved. Since the last century, evidence has been found of a fibrous structure lying above the epidermis (constituting the cuticle) and of another one lying below it (constituting the supporting structures).

The cuticle, easily examined by physical and chemical methods, was analyzed minutely by Singleton (1) and by Maser and Rice (4), who found 80% of it to be proteins (with a high content of hydroxyproline, glycine, and alanine), and 20% to be carbohydrates. These results, as well as the X-ray diffraction pattern, pointed to the presence of collagen. On the other hand, the fibrils observed in the electron microscope have never seemed to show a periodic structure (2–5). It is thought that the collagen molecule of Oligochaeta is twice the weight and length of that of the vertebrates, i.e. it is a dimer of tropocollagen (4).

Although the presence of abundant subepidermal connective tissue in the Oligochaeta has been known since the works of Cerfontaine (6), Bock (7), Schneider (8), and Stephenson (9), no detailed description of it has been available until quite recently. It was only in 1961 that Ruska and Ruska (3) visualized in the electron microscope a 560 A cross-banding in the fibrils of the subepidermal layer of *Lumbricus*, and in 1962 that Van Gansen (10) observed fibrils with a 140 mμ period around the intestine and in the blood plexus of *Eisenia*. In earthworms, the external and internal collagens seem to differ in structure, and the question deserves further study. It is for this reason that I have examined the connective tissue of these animals by as many methods as possible.

MATERIAL AND METHODS

The species studied was *Octolasium complanatum*.

The light, polarizing, and electron microscopes were all used for these researches.

LIGHT MICROSCOPY: Body segments, taken from specimens killed by asphyxiation in sterile water, were fixed in Zenker's solution (for histological examination) or in Duboscq-Brazil's or 10% formaldehyde solution (for histochemical study), embedded in paraffin, and cut serially. The slides were stained by Mallory's technique and hemalum-orange, by the PAS reaction (both with and without previous treatment with saliva for 1 hr at 37° or hyaluronidase for 3 hr at 37°), by paraldehyde-fuchsin after oxidation with sulfuric permanganate, and by the Millon, the Chèvremont and Frederic, the Alcian blue, and the Hale reactions.

POLARIZING MICROSCOPY: Body segments were fixed in 10% formaldehyde or in 70% alcohol, embedded in paraffin, and sectioned serially. Sections were examined in a Leitz microscope furnished with a mercury lamp, and a Berek compensator to determine the sign of birefringence. The retardation values were calculated, with a Brace elliptic compensator, in fluids with different refraction values. Water, 70%, and pure ethyl alcohol, chloroform, α-monobromo naphthalene, and methylene iodide were used alone and together in various proportions. For comparison, the same tests were repeated on alcohol-fixed material after tryptic digestion. Other topochemical tests performed included pepsin digestion and treatment with phenol, picric acid, or clove oil, the sign of birefringence always being checked.

ELECTRON MICROSCOPY: Studies were done on fragments of the body wall (including muscles), the intestine, the blood vessels, and the ventral ganglion chain taken from specimens operated in vivo under dripping glutaraldehyde, pH 7.2. Fixation in phosphate-buffered glutaraldehyde for 2 hr was followed by postfixation in 2% osmium tetroxide in phosphate buffer, pH 7.2, dehydration, and embedding in Araldite. The sections were cut on an LKB 11 ultramicrotome, collected on grids previously coated with Formvar and evaporated carbon, and stained with 1.5% uranyl acetate and lead citrate.

Specimens of fresh tissue were prepared by sectioning with a freezing microtome followed by ultrasonic fragmentation. After being repeatedly washed and centrifuged, part of the material was digested in

trypsin, pH 7.4, for 3–4 hr. The specimens were mounted on Formvar-coated grids and stained with uranyl acetate or phosphotungstic acid. In some cases, negative staining with potassium phosphotungstate was employed.

As a control, collagen from rat tendons was treated by identical methods and observed. The electron microscopes used were the Siemens Elmiskop I of the Department of Anatomy, University of Milan, the Hitachi HS-6 of the Institute of Zoology, University of Siena, and the Hitachi HU-11B of the Electron Microscope Center of the University of Siena.

RESULTS

Histological and Histochemical Characteristics of the Connective Tissue in the Earthworms

Even such simple staining techniques as Mallory's and the hemalum-orange stain clearly show thin basement membranes under the epidermis and around the intestine and blood vessels. Connective tissues abound around the muscles, fibers of which are wrapped in them and tied together by a thick mesh of connective threads, and also around the nervous system, which is wrapped in a thick, compact neural sheath. Regardless of its thickness, the connective tissue demonstrates identical histochemical characteristics. It gives a strongly positive reaction to paraldehyde-fuchsin after oxidation and to the PAS method (both with and without previous treatment with saliva or hyaluronidase); its reaction to the Millon and the Chèvremont and Fréderic techniques is slightly positive; and it is not stained by Alcian blue or Hale's method. These results show that abundant neutral polysaccharide and proteins are present. Thus, the histochemical findings confirm the picture of a fibrous protein network in a polysaccharide matrix.

Characteristics in Polarized Light

All the connective structures of *Octolasium complanatum* are distinctly birefringent in distilled water. The birefringence is consistently positive, in the fibrous structures in relation to the length, and in the basement membranes in relation to the tangent.

The imbibition curves (Fig. 1), calculated with the microscope under uniform lighting conditions and by measuring the retardation values in fluids with different refraction values, were hyperbolic for the various structures examined. Minimal retardation values were obtained for refraction

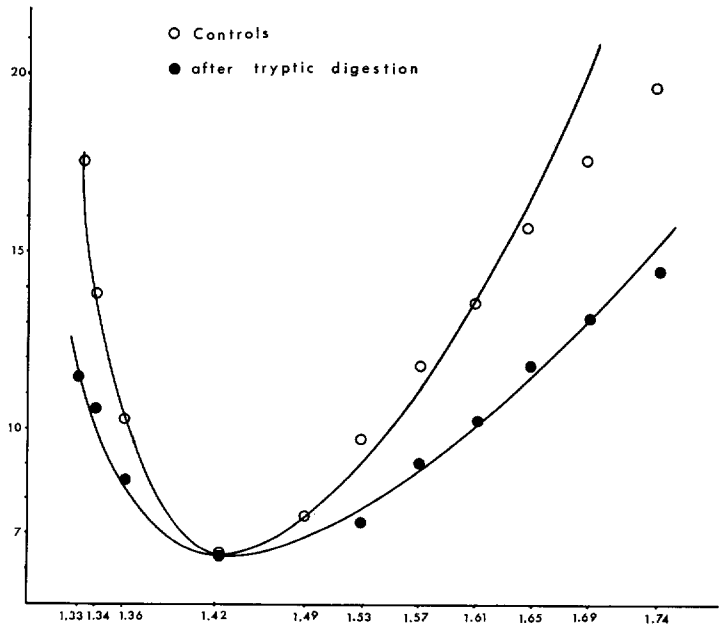


FIGURE 1 Curve of retardation values in perineural collagen of *Octolasium complanatum*. Abscissa: refractive index of the imbibition liquid. Ordinate: retardation in mμ units.

indexes between 1.36 and 1.53 and were found to be around 7–9 A. It was thus demonstrated that the total birefringence of the structures studied is a function of the fluid imbibed and has a double nature: a strong component of textural birefringence overlies an intrinsic birefringence with a value of 7–9 A. Both components are positive. The demonstration of the presence of a composite body with the optic index parallel to the length is evidence of a composite fibrous body consisting of filaments embedded in a substance with a different refractive index.

The enzymatic tests performed subsequently showed that the birefringent connective structures in question are digested by pepsin (after 24 hr at pH 2 and at 37° they have almost disappeared), whereas they resist trypsin: after the sections are treated with 2% trypsin at pH 7.5 and at 37° for 24 hr, the structures still have a positive birefringence. The imbibition curves obtained for this material are similar to those for untreated material, but the maximal retardation values are much lower; that is, the textural birefringence has diminished. Therefore, one can postulate the

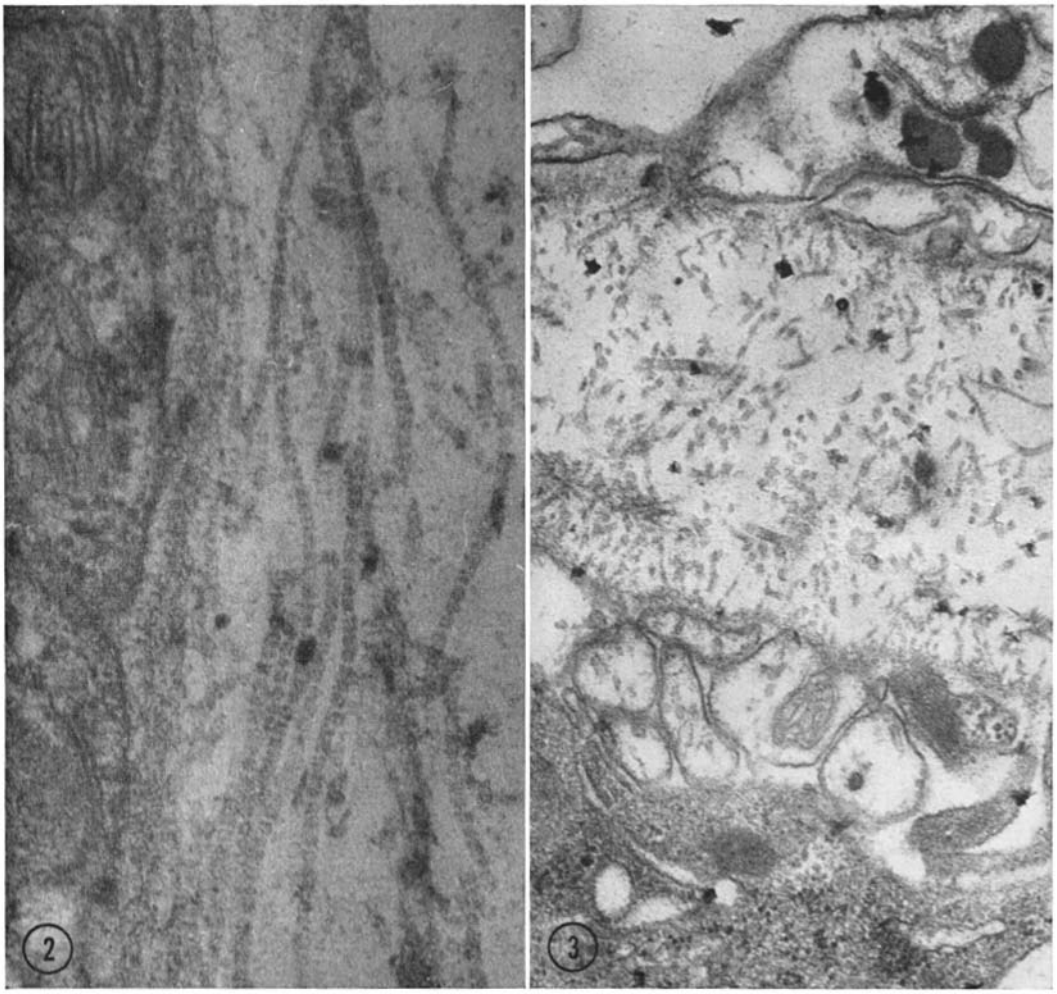


FIGURE 2 Collagen fibrils surrounding the alimentary canal in *Octolasium complanatum*. Fixed and sectioned material, stained with uranyl acetate and lead citrate; Siemens Elmiskop I. × 120,000.

FIGURE 3 Perineural collagen sheath in *Octolasium complanatum*. Fixed and sectional material, stained with uranyl acetate and lead citrate; Siemens Elmiskop I. × 45,000.

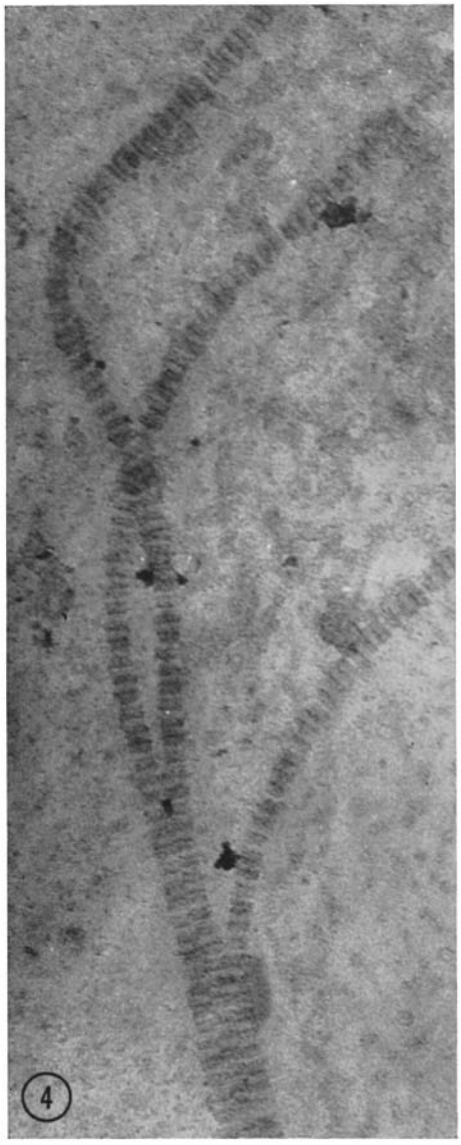


FIGURE 4 Collagen fibrils isolated from *Octolasium complanatum* subepidermal basement membrane. Unfixed and unsectioned material, stained with phosphotungstic acid; Siemens Elmiskop I. × 120,000.

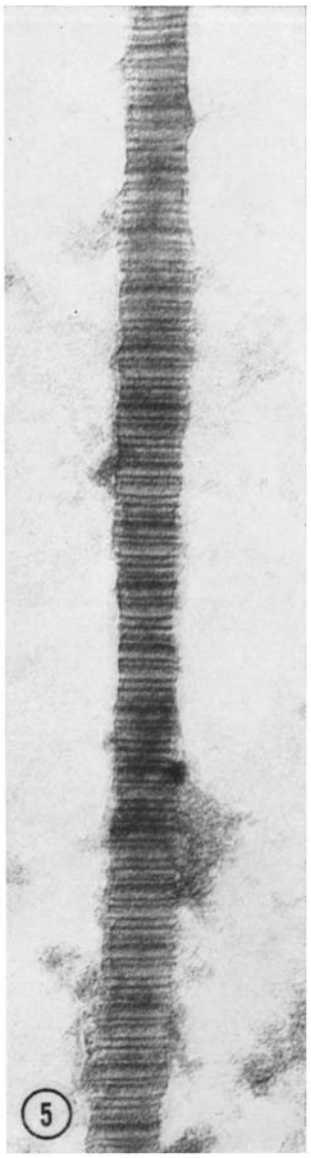


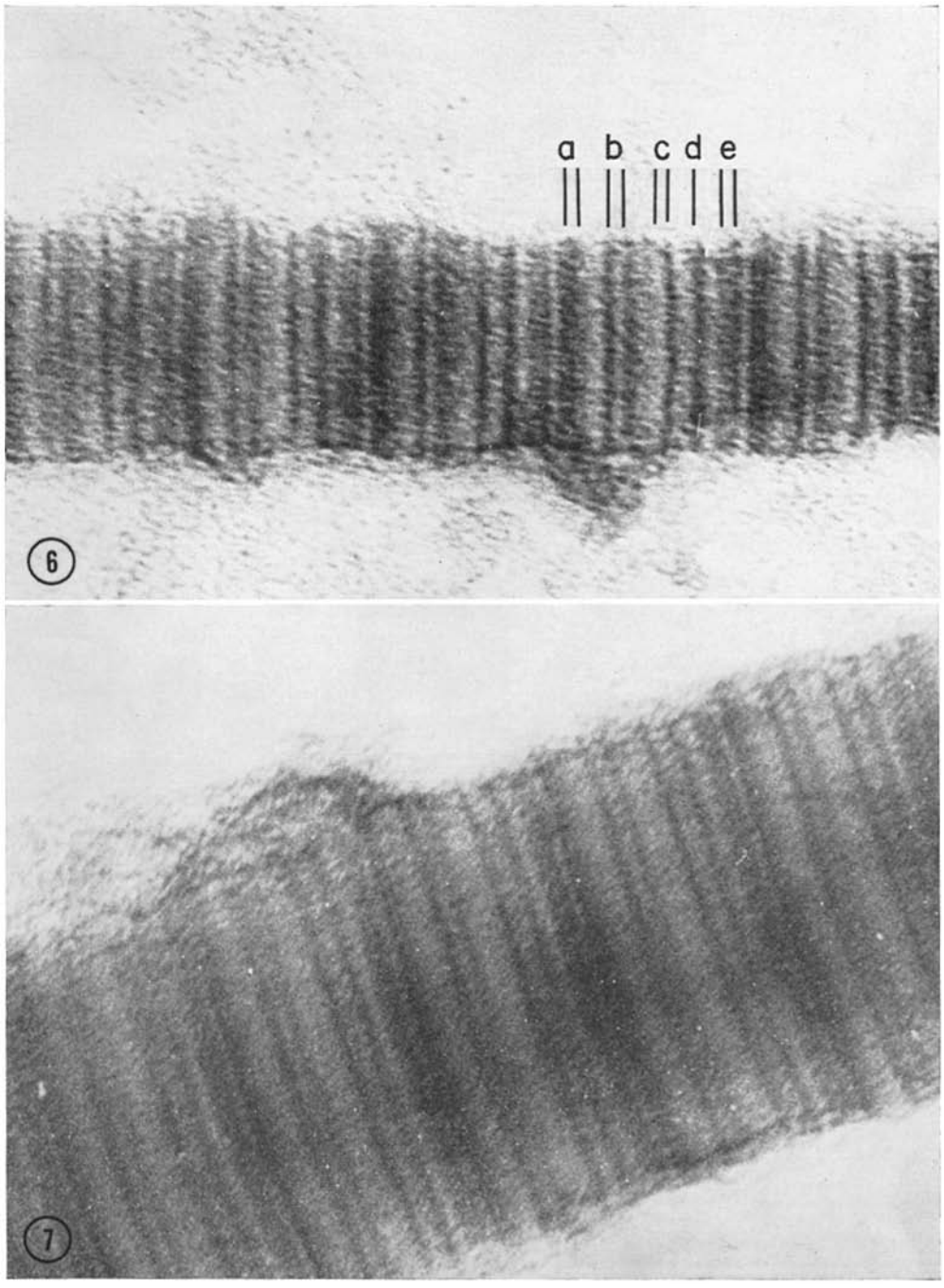
FIGURE 5 Collagen fibrils of *Octolasium complanatum* subepidermal basement membrane. Unfixed and unsectioned material, stained with uranyl acetate; Hitachi HU 11B × 150,000.

existence of trypsin-resistant protein fibers embedded in an amorphous substance that is digested by trypsin. This is further proof of the presence of collagen in the connective tissues examined.

However, certain topochemical tests that are standard for this protein (the inversion of the sign of birefringence after treatment with phenol, oil of cloves, and picric acid) gave unexpected results: negative for phenol (used in 10–15% alcoholic solution as well as water) and picric acid, and weakly positive for clove oil.

Submicroscopic Characteristics

When observed in the electron microscope, all the connective structures of *Octolasium complanatum* described in this paper can be seen to consist of



FIGURES 6–7 Collagen fibrils of *Octolasium complanatum* subepidermal basement membrane. *a*, *b*, *c*, *d*, and *e* indicate the nine bands in the period. Unfixed and unsectioned material, stained with uranyl acetate, Hitachi HU 11b. × 500,000.

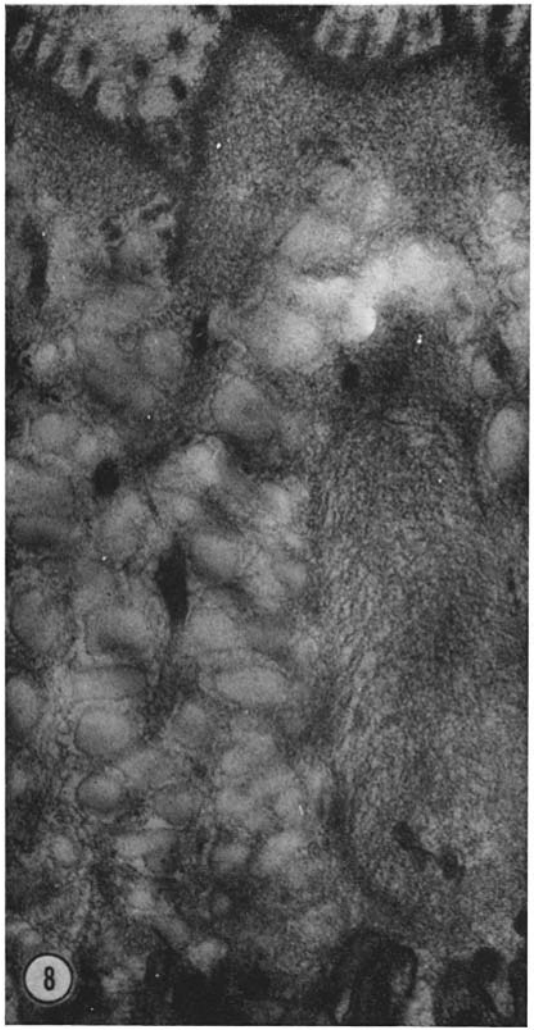


FIGURE 8 Cuticle of *Octolasium complanatum*. Fixed and sectioned material, stained with uranyl acetate and lead citrate; Siemens Elmiskop I. × 45,000.

fibrils grouped into bundles. The weave of the fibrils can be more or less compact. In the basement membranes underlying the epithelium (Fig. 2) or in the threads intertwining among the muscle fibers one finds, at most, 10–12 fibrils side by side in a cross-section, whereas there may be 30–40 juxtaposed fibrils in a cross-section of the neural sheath (Fig. 3) which is more compact and, therefore, poorly resolvable with the light microscope.

Whether sectioned or fragmented, fixed or unfixed, stained with uranyl acetate or with phosphotungstic acid (Fig. 4), the fibrils always show cross-banding with a period of 560 A (data obtained from 130 fibrils measured). Only when the fibrils are photographed immediately after preparation does the period appear slightly longer. It is, therefore, probable that phenomena of imbibition come into play in this situation. It is especially noteworthy that in the rat tendons prepared contemporaneously with the earthworm tissues, the collagen fibril had the usual 640 A period in all cases except, as mentioned above, when photographed immediately after preparation. In such cases, the period was as long as 740 A.

Within the period (Figs. 5–7), the same nine bands typical of mammalian collagen can be resolved quite easily. Following Schmitt and Gross's nomenclature, the first and the second bands (that is, the *a* pair) are very near each other, together covering a space of about 80 A. The *b* pair consists of two very distinct bands, each about 30 A wide. The fifth line (the first of the *c* pair) is a slender one, less than 20 A wide, whereas the sixth line (the second of the *c* pair) is more distinct and about 35 A wide. The seventh (or *d*) line is identical to the sixth. The *e* pair is made up of two lines (the eighth and the ninth) that are very close together and its over-all width is 80 A.

DISCUSSION

The epidermal epithelium of the Oligochaeta is covered by a cuticle composed of collagenous fibrils without periodic bands (3, 4, and data on *Octolasium* in Fig. 8). But, contrary to what Van Gansen (10) concluded for weak resolutions, and in agreement with the findings of Ruska and Ruska (3), the present study has demonstrated that a collagen with a period of 560 A is a constituent of the basement membranes, sheaths, and supporting structures. In this internal collagen, nine principal sub-bands in the period can be resolved, just as in the two models of better known collagen. The collagen of mammals, however, has a period of 640 A (for the latest data, see papers by Olsen, 11, and Bairati, 12), whereas that of insects has a period of 560 A, as does that of the Oligochaeta (13, 14).

The resemblance of the collagen of earthworms to that of insects goes beyond this. Both collagens are embedded in a neutral polysaccharide matrix; both contain few or no cells and, in polarized light, both give negative reactions to certain topochemical tests which elicit typically positive responses with mammalian collagen.

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