

VISUALIZATION OF POLYSACCHARIDES IN
THE CUTICLE OF OLIGOCHAETA BY THE
TRIS 1-AZIRIDINYL PHOSPHINE OXIDE METHOD

Demonstration of 62.5 and 185 Å
Periodicities in Cuticular Fibers

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INTRODUCTION

The cuticle of oligochaets (worms) is a fibrous structure which overlies the epidermal cells (10, 13). Cuticular fibers, as evidenced by wide-angle X-ray diffraction pattern (1-4, 7, 25), amino acid analysis (18, 36, 38), optical rotation (19), and collagenase and trypsin action (19), are shown to contain collagen. Noncuticular collagen of oligochaets in the electron microscope image has 560 Å periodicities (6, 33), but until now no report has demonstrated the presence of these periodicities in the cuticular collagen (6, 37). Cuticular fibers are composed of approximately 80% protein and 20% nonnitrogenous carbohydrates (31). Maser and Rice (20) have suggested that oligochaet cuticular collagen contains dimers of tropocollagen bound by carbohydrates which obscure the ultrastructural pattern by preventing protein side groups from reaction with heavy metals. The significance of carbohydrates bound to cuticular fibers in oligochaets has not, however, been experimentally tested. Nor have any of the molecular models proposed by chemists for the collagen-carbohydrate linkage been morphologically verified. Cuticular fibers fixed by standard chemical methods are uniformly dense in electron micrographs, and no distribution of carbohydrates can be inferred from them.

Since tris 1-aziridinyl phosphine oxide (TAPO) has a strong chemical affinity for polysaccharides (11, 39), we adopted the method of prefixation

with TAPO mixtures (11, and unpublished results) for the study of the distribution of carbohydrates in the cuticle of oligochaets. The TAPO method visualized more structural components of the cuticle than did standard methods; the structural components visualized by TAPO were analyzed by cytochemical techniques. Lead and uranyl staining and cytochemical techniques revealed a crossbanding in the cuticular fibers which had a periodicity compatible with that of the noncuticular oligochaet collagen.

MATERIALS AND METHODS

The following species were used: *Tubifex tubifex* (Mull.), *Eisenia foetida* (Sav.), *Lumbricus terrestris* (L.), *Enchytreus albidus* (Henle), and *Octolasion complanatum* (Dugès). The animals were selected to represent both aquatic and terrestrial biotopes and to cover the greatest possible range of variations in size. Where possible, animals belonging to the same species were collected from different biotopes. Apparently healthy specimens were divided before fixation into three groups, the first being anesthetized with magnesium chloride (9), the second with 7% ethanol, and the third by cold (0°C). Anesthetized worms, either *in toto* or minced with sharp scissors, were prefixed in one of the following prefixatives dissolved in phosphate buffer (0.1 M; pH 7.2): (a) 2.5% glutaraldehyde (28); (b) 1% acrolein; (c) 1% TAPO (39) (Polysciences, Inc., Warrington, Pa.); (d) 1% acrolein and 1% TAPO (11). Methylene chloride was evaporated from the original solution furnished by the producer (80% TAPO and 20% methylene chloride) at 32°C

under low pressure (20 mm Hg); when bubbling ceased, bidistilled water was added to make a 5% TAPO solution. This solution was stored at 0°C and used to prepare the indicated fixatives. The times of prefixation were: for solution (a) 2 h; for solutions (b), (c), and (d) from 2 to 12 h. The mixture of acrolein and TAPO was left at room temperature for 1 h before use. All prefixed worms were postfixed in 4% unbuffered OsO₄ for 12 h. One portion of the worms was fixed exclusively in 1.3% OsO₄ buffered with *s*-collidine (8). All fixed worms were treated overnight with 0.5% uranyl acetate solution in Michaelis buffer (pH 5.8) at room temperature (12-14), then embedded in Vestopal W (27) (lot 68, Madame Martin Jaeger, Genève, Switzerland). Method (a) and fixation with osmium tetroxide alone are referred to in a further text as classical fixation methods. Ultrathin sections were stained with lead citrate and uranyl acetate at room temperature (24, 34, 35) or with 2.5% uranyl acetate in absolute ethanol at 60°C for 3-6 h (15). Some sections were oxidized with periodic acid (E. Merck AG, Darmstadt, Germany) for 25 min, then treated with thiosemicarbazide (E. Merck AG) for a period from 12 to 72 h. The final reaction was carried out either with vapors of osmium tetroxide for 2 h according to Seligman et al. (30) or with silver proteinate (E. Merck AG) for 30 min according to Thiéry (32). No information is available on the influence of TAPO on the course of Seligman's or Thiéry's cytochemical reactions, since free aldehyde groups of acrolein can also give artifactual reactions with cytochemical reagents. Therefore, we carried out thiosemicarbazide treatment and silver proteinate staining after omission of oxidation with periodic acid of sections of acrolein-TAPO-osmium tetroxide-fixed material (see Table II). The effects of the omission of reaction either with thiosemicarbazide or with silver proteinate in periodic acid-oxidized sections of similarly fixed material were also evaluated. The effects of unspecific staining with silver proteinate were evaluated at high magnifications (about × 300,000). Procedures consisting of the omission of oxidation with periodic acid in the material fixed by classical methods do not constitute valid controls since osmium bound to tissue structure is apt to react with thiosemicarbazide and consequently give positive reaction with silver proteinate. However, in the case of acrolein-TAPO-osmium tetroxide-fixed material, no such unspecific binding of thiosemicarbazide to osmium occurred. Other controls were done by evaluating the effects of the omission of reaction either with thiosemicarbazide or with silver proteinate in sections oxidized with periodic acid (Table II). The silver proteinate staining could be run for 20-30 min without causing unspecific staining.

Some sections were oxidized with periodic acid for 25-30 min and treated for 1 h with 1% phosphotung-

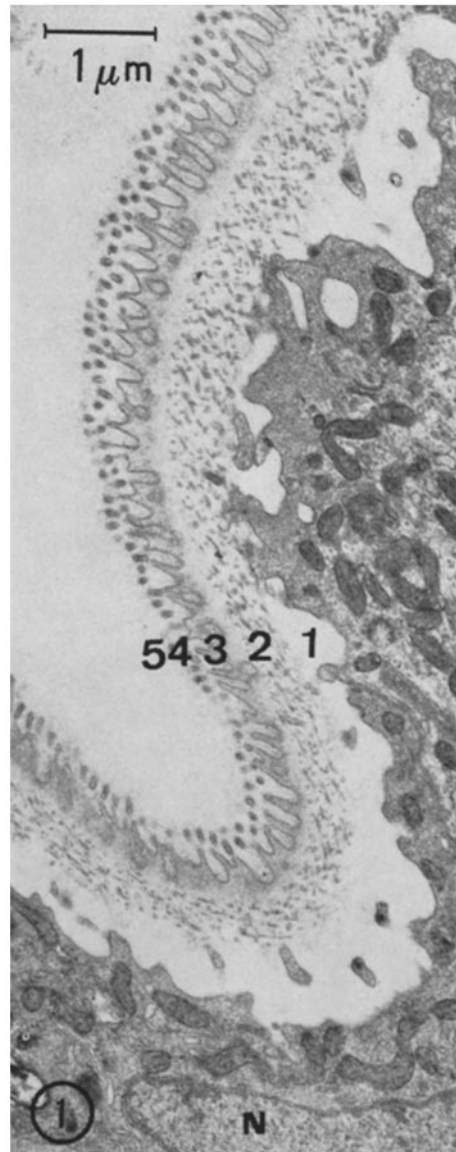


FIGURE 1 *T. tubifex* prefixed with glutaraldehyde and postfixed with OsO₄. The electron micrograph shows the relation between the dermal epithelial cells and the cuticle. Numbers indicate layers of the cuticle. 1, periepithelial space which after application of the glutaraldehyde-osmium tetroxide method is electron transparent; 2, fibrillar layer composed of cuticular fibers embedded in the matrix; both fibers and the matrix are of medium electron density; 3, the epicuticle with electron-opaque cortical layer; 4, ellipsoidal bodies of uniform electron density; 5, external filamentous layer seen here as traces of filamentous material. N, nucleus of the epithelial cell. Lead citrate, uranyl acetate staining at room temperature. × 15,000.

stic acid (PTA) dissolved in distilled water (5, 16, 17, 21, 22).

Siemens Elmiskop I A and 101 electron microscopes were used throughout this study. Care was taken to compensate the astigmatism of the instruments within $0.1\ \mu\text{m}$ with photographic control of the compensation. We determined the magnification of the electron microscopes using the grating replica.

RESULTS

The structure of the cuticle in the species being studied, fixed by classical methods, was compatible with a general pattern found by previous investigators (9, 10, 23, 26). The cuticle is composed of five layers (Figs. 1 and 2).

Figs. 1 and 2 show the pronounced differences in the cuticular structures after fixation by classical methods and by the acrolein-TAPO-osmium tetroxide method. The differences are presented diagrammatically in Table I. It is seen that the acrolein-TAPO-osmium tetroxide method visualizes more structural components of the cuticle

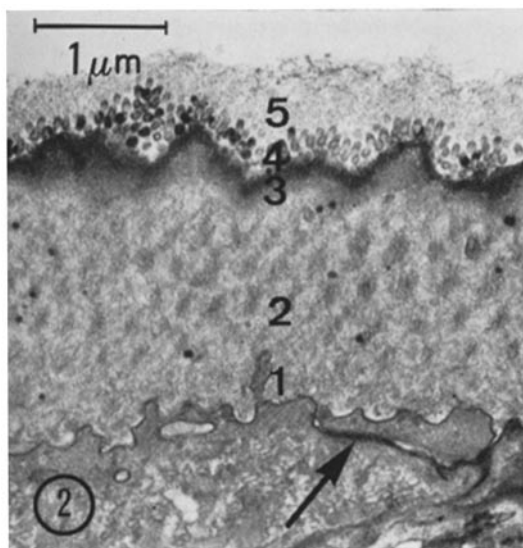


FIGURE 2 *T. tubifex* fixed by the acrolein-TAPO-osmium tetroxide method. As compared with Fig. 1, membranous organelles of epithelial cells are less clearly visible but the layers of the cuticle are better defined morphologically and are more electron opaque. No electron-transparent spaces are seen in the cuticle. The arrow indicates the junctional complex between dermal epithelial cells. After application of the acrolein-TAPO-osmium tetroxide method this complex always contained electron-opaque material. Lead citrate, uranyl acetate staining at room temperature. $\times 17,900$.

than classical methods. No empty spaces are seen in the layers of the cuticle after application of the acrolein-TAPO-osmium tetroxide method (Fig. 2). Among structures visualized only by the acrolein-TAPO-osmium tetroxide method are: a periepithelial layer $250\ \text{\AA}$ thick, closely apposed to the external leaflet of the plasma membrane of epithelial cells (Fig. 3); substructure of the cuticular fibers (Fig. 4); differentiation of ellipsoidal bodies into two populations, one containing an electron-opaque core and another with an electron-transparent core (Fig. 2), could be observed. The acrolein-TAPO-osmium tetroxide method visualized to a higher degree the structures visible also in specimens fixed by classical methods such as the filamentous matrix of the fibrillar layer and the external filamentous layer covering the ellipsoidal bodies.

Each cuticular fiber, in the acrolein-TAPO-osmium tetroxide fixed material, is composed of an electron-opaque peripheral zone and an electron-transparent central core. The peripheral zone of longitudinally cut lead and uranyl-stained cuticular fibers is composed of several layers of filaments. Deeper layers of longitudinally cut cuticular fibers contain filaments regularly spaced at $62.5 \pm 5\ \text{\AA}$ intervals. A crossbanding of the periodicities of $185 \pm 35\ \text{\AA}$ can also be seen.

We observed species-dependent variability of the acrolein-TAPO-osmium tetroxide fixed cuticular structures (see Table II). The periepithelial layer was consistently observed only in *T. tubifex*; 62.5 and $185\ \text{\AA}$ periodicities were found reproducibly in *T. tubifex*, *E. foetida*, *L. terrestris*, and *O. complanatum* while in *E. albidus* the periodicities were found only in 800 – $1,000\ \text{\AA}$ thick cuticular fibers. Other structures undergoing species-dependent variability are listed in Table II.

Cuticles fixed by acrolein alone (method *b*) and by TAPO alone (method *c*) were poorly preserved.

The method of Seligman et al. (30) gave comparable results in cuticles fixed by method (*a*) and method (*d*) (see Table I). No substructure of the main cuticular components, however, could be revealed by this method.

The method of Thiéry (32) gave coarse silver granules over the cuticle fixed by the classical method. Cuticles fixed by the acrolein-TAPO-osmium tetroxide method and stained by Thiéry's reaction showed finer silver granules when compared with those of classical methods, but the

TABLE I
Effects of Different Techniques on the Visualization of Structural Components in *Oligochaeta* Cuticle

Classical methods			TAPO method						
Osmium tetroxide fixation	Glutaraldehyde-osmium tetroxide fixation		Lead uranyl stained	Seligman's reaction	Thiéry's reaction	PTA			
-	-	+	+++	++	++	+++	Periepithelial layer	Visualization of layers of the cuticle	
+	+	++	+++	++	+++	+++	Fibrillar layer		
+	++	+	+++	+	++	+++	Epicuticle		
+	++	-	+++	-	-	+	Ellipsoidal bodies		
+	+	+	+++	+	++	+++	External filamentous layer		
-	-	-	+++	-	+++	+++	Differentiation into peripheral zone and the central core	Cuticular fibers	
-	-	-	+	-	+++	-	62.5 and 185 Å periodicities		
-	-	-	Masked	-	+++	-	Filaments in register with crossbanding		
+	+	-	+++	+	+	++	Density of the matrix		

- Not visible.
+ Low contrast.
++ Medium contrast.
+++ High contrast.

resolution of silver proteinate staining was always low. Thiéry's reaction visualized the differentiation of cuticular fibers into a peripheral zone and a central core. A superficial layer of the longitudinally cut peripheral zone of cuticular fibers after silver proteinate staining (32) was irregularly covered by silver granules. Deeper sections of this zone showed crossbanding of two types (Fig. 5): (a) linearly arranged deposits of silver granules spaced at 60-65 Å intervals, and (b) broad regular bands with a center-to-center distance of about 185 Å. Only a small part of the structures revealed by lead and uranyl staining in the matrix of cuticular fibers fixed by the acrolein-TAPO-osmium tetroxide method reacted with silver proteinate (32) (Table I). Among the reacting structures were filaments oriented perpendicular to the cuticular fibers, in register with their dense bands. The periphery of the ellipsoidal bodies and the external filamentous layer gave positive Thiéry's reaction.

The results of procedures in which certain steps

of the cytochemical staining were omitted in order to be able to evaluate better the results of Thiéry's reaction as applied to acrolein-TAPO-osmium tetroxide-fixed cuticles are summarized in Table III. It can be seen that periodic acid oxidizes all metallic deposits, and aldehydic groups introduced by the technical procedure were sparsely dispersed only over the epicuticle. Other controls show that the majority of silver granules is bound to thiosemicarbazide, which reacted with oxidized vic-glycol groups since direct staining with silver proteinate does not augment the electron density in cuticular structures.

DISCUSSION

The acrolein-TAPO-osmium tetroxide fixation furnished a higher contrast and a higher resolution of cuticles than classical methods of fixation of oligochaets for electron microscopy. As well, silver proteinate reaction (32) gave a higher resolution in the acrolein-TAPO-osmium tetroxide-fixed cuticles than in those fixed by classical methods.

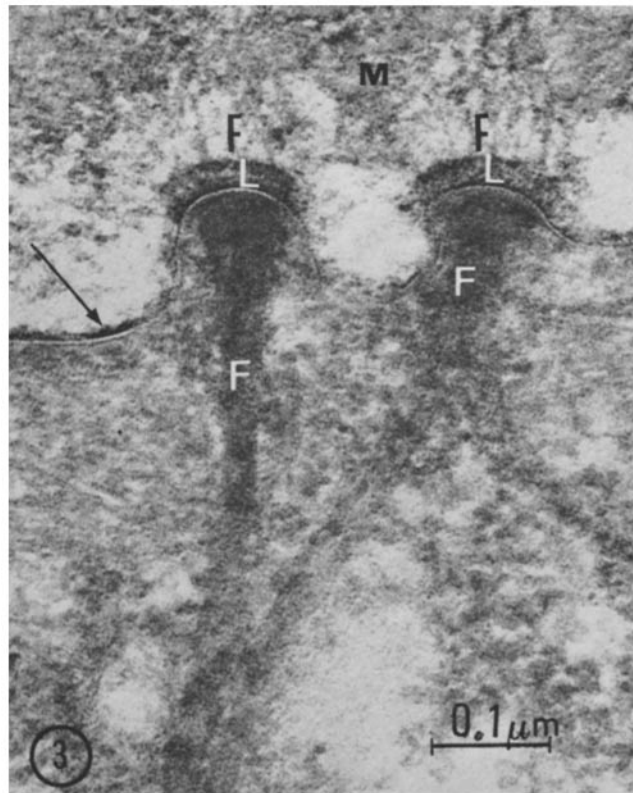


FIGURE 3 *T. tubifex* fixed by the acrolein-TAPO-osmium tetroxide method. The electron micrograph shows two half-desmosomes. In the periepithelial space the uniform dense layer (arrow) is closely apposed to the external leaflet of the plasma membrane of epithelial cells. A similar layer is seen in the half-desmosomes together with other layered material of alternating electron density (*L*). Externally to this material, filaments (black *F*) connect the half-desmosomes with the matrix of the fibrillar layer (*M*). Anastomosing filaments (white *F*) anchor half-desmosomes deeply in the cytoplasm of the epithelial cell. Stained with 2.5% uranyl acetate in absolute ethanol for 6 h at 60°C. $\times 152,000$.

We observed many new structural components of the cuticle in acrolein-TAPO-osmium tetroxide-fixed oligochaets.

A considerable part of the new structural information concerned the cuticular fibers. We observed that they were composed of an electron-opaque peripheral zone and an electron-transparent central core. The possibility does exist that differences in electron density are due to inadequate penetration of the TAPO mixture into the cuticular fibers. If this were so, then subsequent cross-reactions between molecules of cuticular fibers, fixatives, and various staining reagents could in themselves lead to the differences in electron density revealed in the cuticular fibers. Since similar differences are visible in cuticular fibers also after silver proteinate reaction, which

does not strongly depend on the presence of TAPO in biological structures (32), and since there is an abrupt fall of electron density at the interface between the peripheral zone and the central core, the existence of these components *in vivo* is not excluded. Only the application of the acrolein-TAPO-osmium tetroxide method permits the visualization of the crossbanding of cuticular fibers. Such a crossbanding has never been observed in the electron microscope, and the spatial order in cuticular fibers has been deduced only from X-ray crystallographic studies (1-4, 7, 25).

It is difficult to compare structures evidenced in acrolein-TAPO-osmium tetroxide-fixed, lead- and uranyl-stained material with similarly fixed cuticles which are silver proteinate stained. The silver proteinate reaction revealed only a part of



FIGURE 4 *E. foetida* fixed by acrolein-TAPO-osmium tetroxide method. Cuticular fibers, as seen in cross sections, are composed of a peripheral electron-opaque zone (arrows) and an electron-transparent core (c). Lead citrate and uranyl acetate staining at room temperature. $\times 50,000$.

the cuticular structures, namely, those containing polysaccharides with free *vic*-glycol groups. On the other hand, a majority of the cuticular structures could be revealed by lead and uranyl staining. Cuticular structures not revealed by silver proteinate staining and visible only after lead and uranyl staining may also contain polysaccharides, but their *vic*-glycol groups may be masked or absent.

Staining with PTA, in spite of controversies concerning its specificity (29), gives useful information.

X-ray diffraction (1-4, 7, 25) and chemical analysis (18, 19, 36, 38) have unequivocally shown that cuticular fibers contain collagen; the only subject for speculation was the location of the collagen in the cuticular fibers. We have not been able to furnish any data in support of a hypothesis on this location. The "insertions," (unpublished results) that unique structure which would account for the end-to-end linkage between proteins and carbohydrates, as suggested by Maser and Rice (20), are spaced by intervals that are larger than the length of the tropocollagen molecule, while periodicities of transverse banding reported in this note are too small. Only the peripheral zone of

TABLE II
*Species-Dependent Variability of the Acrolein-TAPO-Osmium Tetroxide-Fixed Cuticular Structures**

Species	Periepithelial layer	Cuticular fibers			External filamentous layer	
		62.5 and 185 Å periodicities	Mean diameter	Ellipsoidal bodies‡	Thickness	Overlapping ellipsoidal bodies
			μm	μm	μm	
<i>T. tubifex</i>	Consistently visible	Visible in a majority of fibers	0.13	0.16	0.45	Yes
<i>L. terrestris</i>	Occasionally visible	Visible in a majority of fibers	0.10	0.10	0.40	Yes
<i>E. foetida</i>	Occasionally visible	Visible in a majority of fibers	0.09	0.15	0.15	Yes
<i>O. complanatum</i>	Not visible	Visible in a majority of fibers	0.13	0.10	—	No; loosely arranged between ellipsoidal bodies
<i>E. albidus</i>	Not visible	Visible only in 800-1,000 Å thick fibers	0.40	0.21	—	No; tightly packed between ellipsoidal bodies

* Differentiation of cuticular fibers into peripheral zone and central core as well as the composition of the epicuticle of alternating electron-opaque and electron-transparent layers was visible in all studied species.

‡ Mean length of the main axis.

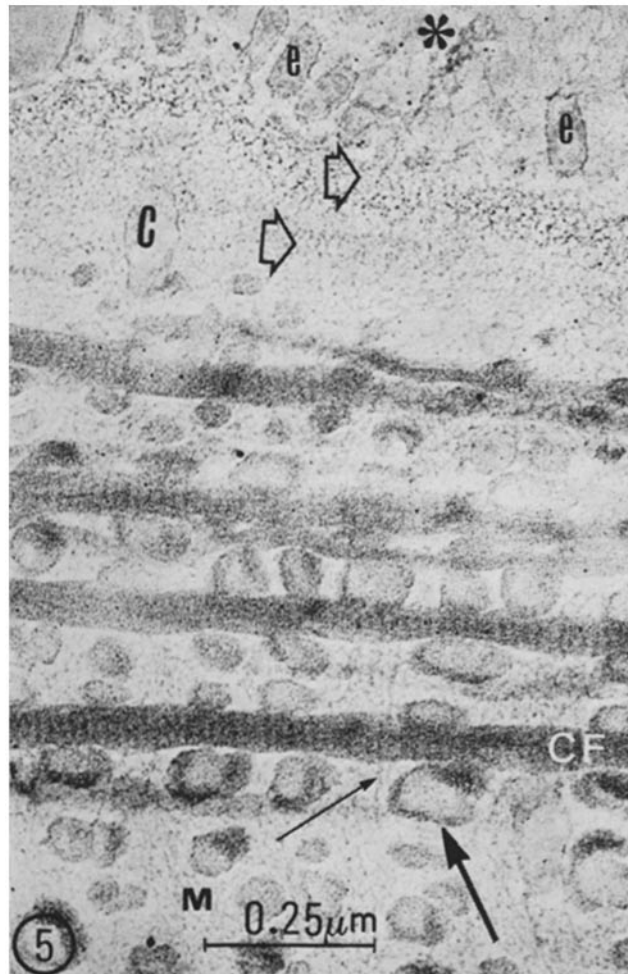


FIGURE 5 *T. tubifex* fixed by the acrolein-TAPO-osmium tetroxide method. Sections were oxidized for 25 min with periodic acid, left to react with thiosemicarbazide for 72 h, and then stained with silver proteinate for 30 min. Cuticular fibers (CF) cut longitudinally show the presence of transverse banding of regular periodicity. Some filaments of the matrix (small arrow) are seen to be in register with dense bands on cuticular fibers. Cross sections of cuticular fibers (large arrow) show the presence of deposits of silver granules predominantly over the peripheral zone of the fiber. Granules of silver are also seen external to the plasma membrane of the cytoplasmic process (C). Epicuticle shows deposits of silver granules over two distinct layers (empty arrows). Silver granules mark the periphery of ellipsoidal bodies (e). Large accumulations of silver granules are seen just to the right of the asterisk over the external filamentous layer. $\times 90,000$.

cuticular fibers reacted with silver proteinate. If the division of cuticular fibers into a peripheral zone and a central core reflected the *in vivo* state, the only possible location for collagen would be the central core. However, none of the techniques used in this study has revealed any periodicity typical for oligochaet noncuticular collagen in the central core of the cuticular fibers. It is possible

that the lack of penetration of the TAPO mixtures into the central core disallows cross-binding with heavy metals. The periodicities of the crossbanding of cuticular fibers (62.5 and 185 Å) when multiplied by the factor 9 or 3 give the value of 560 Å, which corresponds to the main period of the noncuticular collagen of oligochaets (6, 33). This smaller sub-periodicity, however, might be explained in other

TABLE III
Tests of the Influence of Acrolein-TAPO-Osmium Tetroxide Fixation on Thiéry's Reaction

	Procedures and results			
	Oxidation with periodic acid	Omitted	Done	Done
Treatment with thiosemicarbazide	Done	Omitted	Omitted	Omitted
Silver proteinate staining	Done	Done	Omitted	Done
	Scarce irregular deposits over epicuticle	Complete loss of electron density	Complete loss of electron density	No change in the distribution of electron density

ways, particularly on the basis of different kinds of alignment of the molecules within the cuticular fiber.

This note furnishes another example of the applicability of the TAPO method for the visualization of polysaccharides in biological structures. It is interesting to note that the TAPO mixture of the same composition as used for the yeast cell wall (11, and unpublished results) could also be applied successfully in the group of organisms which evolutionarily are quite distant from yeasts.

SUMMARY

The acrolein-TAPO-osmium tetroxide fixation visualized new structural components in the cuticle of several species of Oligochaeta. The application of the method caused an increase in the electron density of the cuticular structures which, according to the results of the cytochemical reactions, contain polysaccharides. In the material fixed by acrolein-TAPO-osmium tetroxide, the lead and uranyl staining revealed a larger number of cuticular structures than in the reaction of Thiéry. Silver proteinate staining of acrolein-TAPO-osmium tetroxide-fixed cuticular fibers showed crossbandings of 62.5 and 185 Å periodicities in the peripheral zone. Such periodicities could be revealed occasionally by lead and uranyl staining in similarly fixed material. Even if the periodicities of cuticular fibers were geometrically compatible with the 560 Å main period of non-cuticular collagen of oligochaets, it would be possible to explain these periodicities on the basis of a different kind of molecular alignment.

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