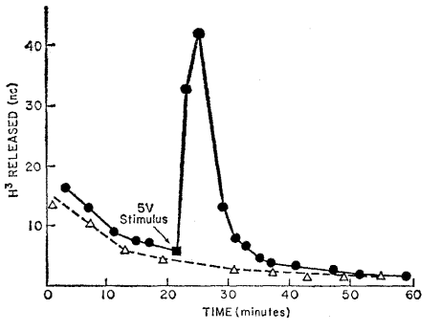

Fig. 1. Release of H^3-norepinephrine from a coronal section of rat brain, induced by a 5-volt, 60-cycle electrical stimulus delivered for 60 seconds, compared with unstimulated efflux from a similar slice. This pattern of release was typical of 18 stimulated brain slices. Closed circles, with stimulation; open triangles, without stimulation.

seconds, were almost as efficient as higher voltages (5 to 10 volts). For stimuli of 4 volts, applied for intervals of 15 to 60 seconds, there appeared to be a linear relation between duration of stimulation and release of norepinephrine. When prolonged stimulation of 10 to 12 volts was used, release of H^3-norepinephrine was apparent only during the first 2 to 4 minutes.

Stimuli of 5 volts, applied for 60 seconds, raised the temperature of the perfusion chamber to a maximum of about 37.5°C. When the bath temperature was raised from 37° to 40°C, insignificant amounts of H^3-norepinephrine were released from brain slices. Thus, effects of electrical stimulation do not appear to be due merely to heating. Efflux of C^{14} from brain slices saturated with C^{14}-urea was not altered by electrical stimulation, which indicates that enhanced diffusion did not play a role in the release of norepinephrine.

Table 1. Release of tritium from tissue slices. Data are mean differences (± standard error of mean) between base line of tritium washout and maximum release following a 5-volt, 60-cycle electrical stimulus for 60 seconds. KCl was used without electrical stimulation, and the data given represent the maximum release obtained after changing to a medium containing 33 m*M* KCl. ES, electrical stimulation.

Brain		Heart	
H^3 released (nc)	*N*	H^3 released (nc)	*N*
Electrical stimulation			
30.3 ± 2.0	18	22.4 ± 2.5	6
Ca-free medium + ES			
11.8 ± 1.0	6	4.5 ± 2.0	6
Chlorpromazine (40 μg/ml) + ES			
13.7 ± 2.2	6		
Pentobarbital (100 μg/ml) + ES			
		7.3 ± 1.0	6
High KCl (33 mM)			
24.0 ± 1.2	3	38.0	1

Effects of electrical stimulation can be reproduced by high concentrations of potassium in the perfusion medium. Marked release of tritium from brain slices was induced by Ringer media containing 16 to 66 m*M* KCl. Such media are known to depolarize nerve membranes in brain slices (*5*) and to stimulate respiration by the slices (*10*).

Absence of calcium from the medium markedly inhibited release of H^3-norepinephrine by electrical stimulation. Pentobarbital and chlorpromazine appear to inhibit release of H^3-norepinephrine from brain or heart slices by electrical stimulation (Table 1); the effect of pentobarbital on release of tritium was much greater in heart slices. These agents inhibit the increase in respiration that is observed in brain slices during electrical stimulation (*11*).

We have also found that H^3-histamine, H^3-dopamine, and some other amines which accumulate in brain slices in vitro can be released to some extent by electrical stimulation. Thus the present technique may be of some general use in investigation of the biochemistry and pharmacology of depolarization-induced release of central neurotransmitters.

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3 March 1966

Staining Tissue for Light and Electron Microscopy by Bridging Metals with Multidentate Ligands

Abstract. *"Osmium black," a pigment very useful for cytological staining in both light and electron microscopy, may be deposited selectively at the tissue-binding sites of other metal ions by bridging OsO_4 to the tissue-bound metal ion through a multidentate ligand.*

Certain osmiophilic reagents for the selective deposition of osmium black at the sites of enzymes and functional groups in tissue (*1*) have an additional property which is useful for staining tissue. These multidentate reagents have an affinity for metals bound to tissue and retain the ability to react further with the same or other unbound metal by bridging. In our studies, thiocarbohydrazide (TCH), a multidentate organic sulfur ligand, is very useful in this bridging reaction. In its simplest form, the reaction may be depicted as in Fig. 1, where M_1 is the primary and M_2 the secondary metal cation. Thiocarbohydrazide, which has been used with osmium tetroxide for the histochemical demonstration of some oxidized macromolecules (*2*), is able to

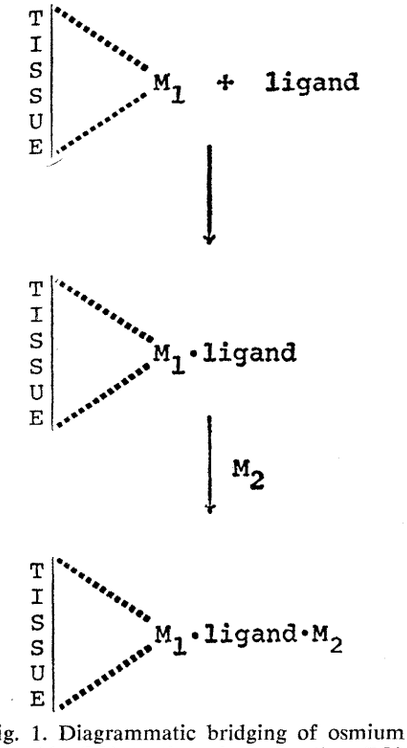

Fig. 1. Diagrammatic bridging of osmium tetroxide (M_2) to tissue-bound cation (M_1) through a multidentate ligand, TCH.

bridge osmium black to many tissue-bound cations. For example, osmium may be bridged to tissue-bound osmium present after fixation with osmium tetroxide. This bridging process, which we call the OTO method, is especially useful in electron microscopy and results in the enhancement of osmiophilic lipid components of ultrastructure (*3*). The OTO method increases contrast and resolution of membranes, droplets, granules, and other ultrastructures to such a degree that it may offer an alternative to the lead and uranium staining methods (Fig. 2). Its distinct advantage is the absence of contamination encountered with heavy-metal staining, and absence of staining of other than osmiophilic structures, such as glycogen.

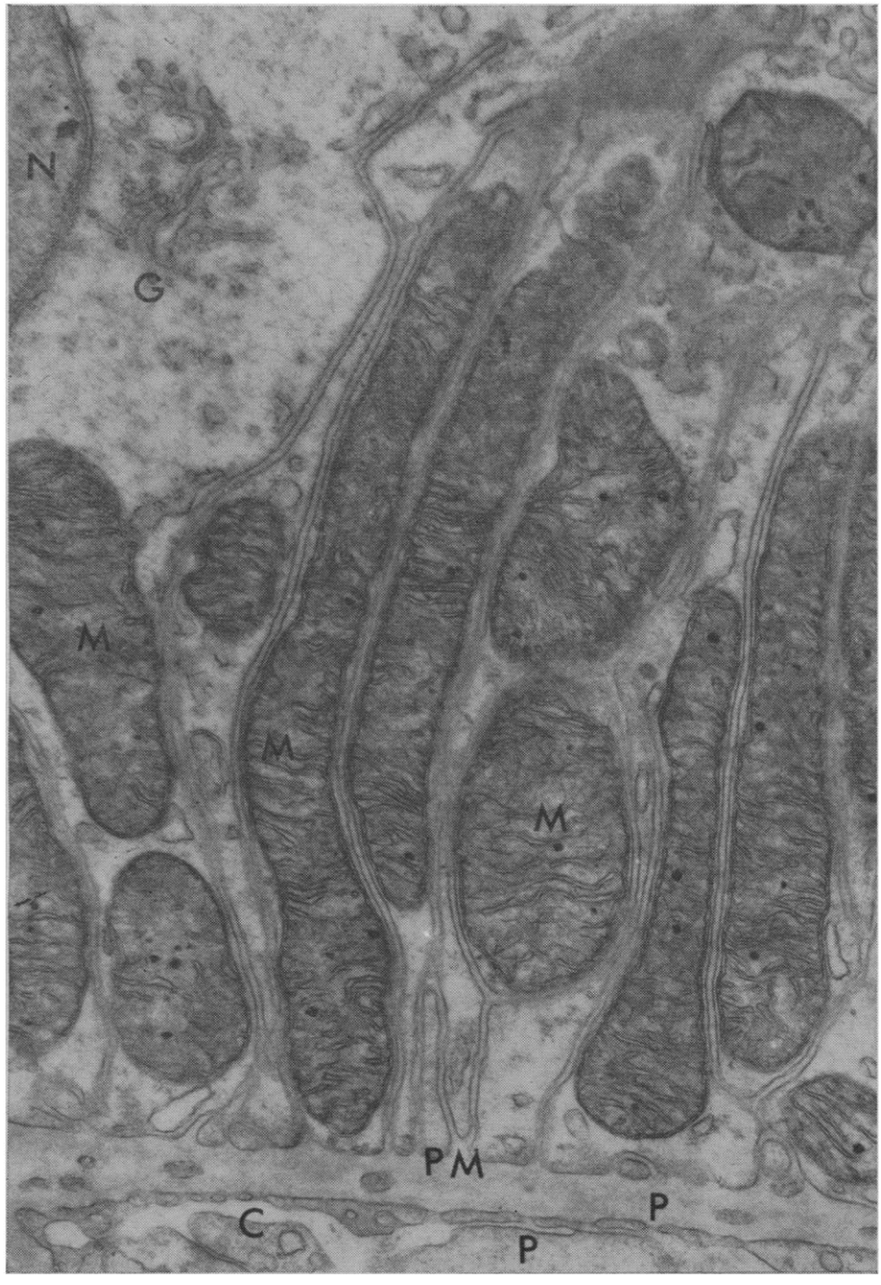

Fig. 2. Electron micrograph of part of a cell of the distal convoluted tubule stained by the OTO method. Osmium tetroxide–fixed rat kidney, embedded in araldite, cut in ultrathin sections placed on gold or inert metal grids, treated with a 1 percent aqueous solution of TCH at 50°C for 1 hour, washed with hot water several times for 15 minutes each and exposed to osmium tetroxide vapor at 60°C for 1 hour. There is clear delineation of all membranous structures, such as cristae and outer limiting membranes of the mitochondria (*M*), the envelope of the nucleus (*N*), the Golgi complex (*G*), the deep infoldings of the plasma membrane (*PM*), and the thin endothelium of the capillary (*C*) characterized by single-layered membranous pores (*P*). The dense granules in the mitochondria are especially prominent. (× 24,000)

The sites of attachment of cations which are invisible or have low-electron opacity may be demonstrated for light microscopy and electron microscopy (*3*) by bridging osmium to the tissue-bound metal ions. The visualization of uranium-labeled antigen (*4*) is made more prominent in electron micrographs by bridging osmium to the protein-bound uranium with TCH (*5*). As seen in preparations for light microscopy, an osmium black reduction product of OsO_4 (M_2) is deposited at the binding sites of the primary cation (M_1), after treatment with TCH, a wash to remove unattached TCH, and subsequent treatment with osmium tetroxide. Carbohydrazide, (CH) was almost as effective as TCH as a bridging ligand; tetraethylenepentamine and H_2S were much less effective, and 1,5-diaminopentane or hydrazine did not bridge the metals at all.

The sites of attachment of cations of Pd, Os, U, Pb, Hg, Fe, Cr, Cu, Ca, Zn, and Sn have been demonstrated with varying degrees of efficacy in formalin- or glutaraldehyde-fixed kidney sections, impregnated with primary metal, treated with TCH, washed, and then exposed to osmium tetroxide (Figs. 3–5). Sections treated with magnesium or aluminum salts were negative.

When unidentate aryl thiols such as 4-chlorothiophenol, thiophenol, or 2-naphthalenethiol were substituted for the polydentate ligand, TCH, on glutaraldehyde- or formalin-fixed tissue or tissue already treated with osmium tetroxide, a very much weaker reaction occurs. There is some evidence that the reaction with thiophenols may occur by a mechanism other than the bridging mechanism, although these unidentate ligands have been reported (*6*) to bridge metals. When the unidentate thiophenols are bound to tissue by a metal, which is a very good electron acceptor and forms particularly stable complexes, such as palladium, the reaction with the secondary metal cation does not occur at all. This indicates that the mixed ligand complex, tissue-osmium-thiophenol, may release thiophenol when treated with the strong oxidizing agent osmium tetroxide, whereas the less labile tissue-palladium-thiophenol complex does not liberate thiophenol.

To test the relative degree of stain-

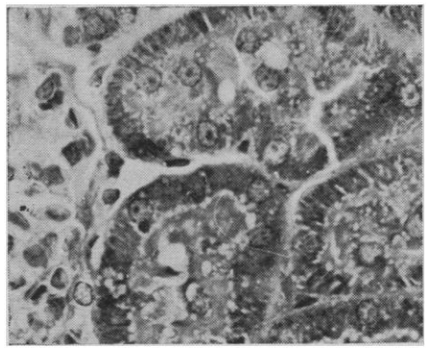

Fig. 3. Bridging of osmium black to palladium. Glutaraldehyde-fixed rat kidney; cut in paraffin sections, hydrated and treated with 0.01 percent K_2PdCl_4 solution for 30 minutes, washed and treated with 1 percent TCH in ethanol for 30 minutes, washed and exposed to osmium tetroxide vapor for 15 minutes at 60°C. Note prominent osmium black nuclear and nucleolar stain in tubular cells and cells in the glomerulus (left) and in the cytoplasm.

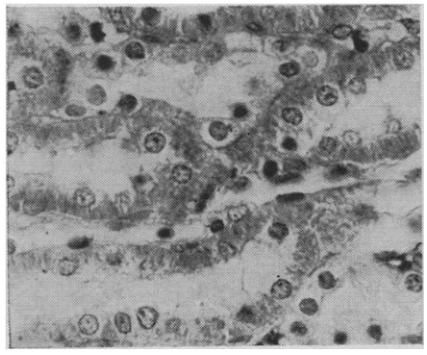

Fig. 4. Bridging of osmium black to mercury. Formaldehyde-fixed rat kidney, cut in paraffin sections, hydrated and treated with a saturated solution of $HgCl_2$ for 1 hour, washed, treated with 1 percent TCH in ethanol for 30 minutes, washed and exposed to osmium tetroxide vapor for 15 minutes at 60°C. Note prominent osmium black stain of cell nuclei and nucleoli as well as light stain of cytoplasm.

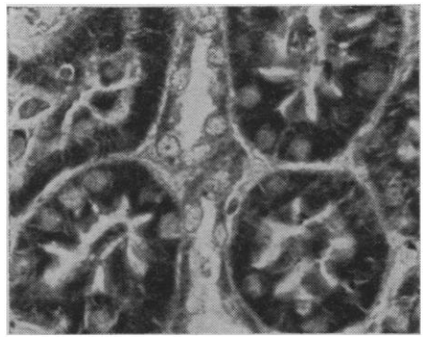

Fig. 5. Bridging of osmium black to uranium. Dark osmium black stain in cytoplasm of tubular cells and virtually unstained cell nuclei. Same fixation as Fig. 4, treated with 1 percent uranyl acetate for 1 hour, washed, and treated with 1 percent TCH in ethanol for 30 minutes, washed and exposed to osmium tetroxide vapor for 15 minutes at 60°C.

ing by the bridging procedure of protein, lipid, carbohydrate, and nucleic acid constituents of tissue, samples representing these constituents were impregnated on small disks of Whatman No. 1 filter paper and subjected to the osmium tetroxide–ligand–osmium tetroxide procedure. Papers were impregnated with approximately 0.3 mg of casein, deoxyribonucleic acid, ribonucleic acid, potato starch, or menhaden oil (Gulf). The papers were then suspended successively in 1 percent OsO_4 solution (½ hour), bridging ligand solution (10 minutes), and 1 percent OsO_4 solution (½ hour), with thorough washing after exposure to each reagent solution. The staining was most pronounced with the oil, very much weaker with protein and nucleic acids, and negative with potato starch. During the course of this treatment, as well as in experiments with tissue and by the study of the appropriate controls, some staining of lipid and very little staining of protein was noted by the initial application of osmium tetroxide. This staining was enhanced to a brown hue by treatment with the bridging ligands TCH and CH. Upon exposure to the final OsO_4 solution, further intensification was observed, and the brown color was replaced by an intense black color.

Although the final black color was approximately equal in intensity on the paper disks, whether TCH or CH was used as the bridging ligand in the glutaraldehyde-fixed kidney sections, the TCH gave slightly greater contrast than CH. This was also true with tissue fixed in osmium tetroxide. No differences were noted between formalin- or glutaraldehyde-fixed kidney sections in light microscopy.

The demonstration of the attachment of osmium black to tissue-bound palladous chloride, mercuric chloride, or uranyl acetate by the bridging reaction is shown in kidney sections in Figs. 3–5. In studies on tissue sections, palladous ion as the primary metal could bridge osmium through TCH when the tissues were treated with K_2PdCl_4 solutions of approximately one-hundredth the concentration of the other primary metal-salt solutions. The distribution noted was similar to that of other heavy metals. This great affinity of palladium salts for tissue is not surprising in view of the reported high stability of palladium chelates (*7*).

Osmium may also be bridged through TCH to metals which have been localized in tissue as a result

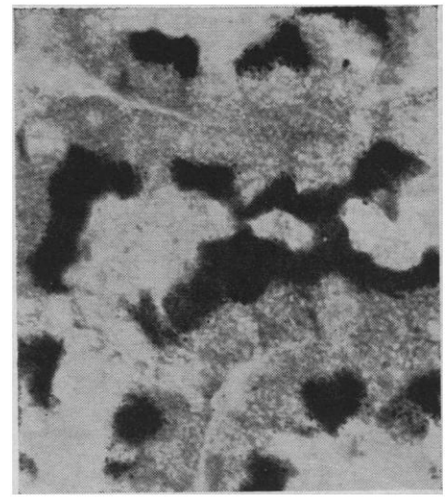

Fig. 6. Aminopeptidase activity in brush border of renal tubules of the rat shown with osmium black by bridging osmium to copper chelate of the azo dye produced by the histochemical reaction. After glutaraldehyde fixation, cryostat sections were incubated for 15 minutes with L-leucyl-4-methoxy-2-naphthylamide and fast blue B. The sections were chelated by exposure for 5 minutes to 0.1*M* cupric sulfate, washed thoroughly in water, treated with 1 percent aqueous solution of TCH for 15 minutes, washed, and exposed to osmium tetroxide vapor for 15 minutes at 60°C. Sections were dehydrated and mounted in Permount. Note sharp limitation of aminopeptidase activity to the brush border.

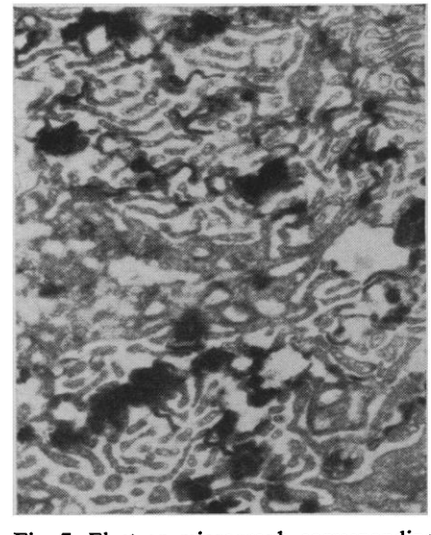

Fig. 7. Electron micrograph corresponding to Fig. 6. Cryostat sections (40 μ) were used and 7 percent sucrose was used in all washes and incubation medium for aminopeptidase. Sections were embedded in araldite, and ultrathin sections on gold grids were treated with 1 percent TCH in water for 1 hour at 50°C, washed, and exposed to OsO_4 vapor at 60°C for 1 hour. Note deposits of osmium black due to aminopeptidase activity in and on the microvilli of the brush border of the renal tubular cell. (× 22,000)

of a histochemical reaction. For example we have bridged osmium through TCH to copper or mercury which is localized by chelation to the azo dye formed at the sites of aminopeptidase activity (*8*). The labeling of sites of aminopeptidase activity in rat kidney with osmium black is shown in a light micrograph, Fig. 6, and an electron micrograph, Fig. 7.

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Mycotoxicoses: Toxic Fungi in Tobaccos

Abstract. *Cigarette, cigar, and pipe tobaccos contain* Alternaria *spores and dematiaceous mycelia, with cigarette tobaccos being more heavily laden. A smoke aerosol generated from hay on which the fungus had been cultured caused, among other pathologic changes, pulmonary emphysema in mice.*

Various constituents of cigarette smoke have been studied as possible causes of pulmonary disorders (*1*), but mycotoxins have not been investigated. Random samples of 252 tobaccos from various brands of cigarettes and 50 each from cigars and pipe mixtures were examined mycologically; two predominating fungal isolates, a species of *Alternaria* and an *Aspergillus niger*, were subjected to preliminary toxicity tests in ICR mice by the oral and smoke aerosol routes.

Stereomicroscopic examination revealed no active fungal proliferation. Microscopic examination indicated past fungal growth, particularly in cigarette tobaccos (Fig. 1) which were considerably more heavily laden with *Alternaria* spores and corresponding dematiaceous mycelia than tobaccos from cigars or pipe mixtures (Table 1). The recovery, by culture, of viable fungi was very low, particularly in cigarette tobaccos.

The *Alternaria* and *Aspergillus niger* were grown at room temperature on Czapek's solution agar in petri plates, and aqueous homogenates were prepared by macerating, in a Ten Broeck tissue grinder, one part of fungal substratum with two parts of distilled water. Each of five mice received daily by stomach tube 1.0 ml of a respective homogenate until death occurred or for a maximum of 3.5 weeks. *Alternaria* homogenate produced progressive hypotonia after 24 hours, subepidermal hemorrhages by the 7th day, anorexia and pronounced cachexia by the 9th day, and death in all mice by 3.5 weeks. *Aspergillus niger* was not as toxic as *Alternaria*. Five control mice that received noninoculated agar homogenate daily for 3.5 weeks remained normal.

Timothy hay adjusted to approximately 20 percent moisture in Fernbach flasks, autoclaved (1 atm for 45 min), was inoculated with fungus and incubated for 4 weeks at 24°C; the culture was air-dried, pulverized, adjusted to approximately 20 percent moisture with 4 percent aqueous glycerol, and tested for smoke aerosol toxicity. Noninoculated, sterilized timothy hay, similarly treated, served as a control. The smoking apparatus consisted of a smoking pipe bowl attached by a piece of gum tubing to a glass tube which extended through a two-hole stopper into the bottom of a glass test tube, 65 by 500 mm. A piece of gum tubing attached to a glass tube extending through the stopper 5 cm into the test tube was connected to a vacuum source. Mice were placed in the smoke chamber, and, after a slight negative pressure was attained, the fungus substratum in the pipe was ignited and smoke was allowed to fill the chamber. Exposure time was 7 seconds daily until death occurred, or for a maximum of 466 days.

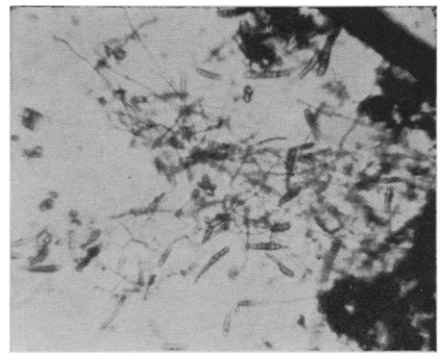

Fig. 1. Proliferative dematiaceous mycelia and dictyospores of *Alternaria* species in heavily contaminated cigarette tobacco. KOH preparation (about × 65).

After the 5th month, four of four mice challenged with *Alternaria* smoke developed progressive hypotonia and cachexia; in addition, from the 9th month until death ensued, there was lachrymation, edema, serum exudation with subsequent encrustations, and loss of hair about the face. Gross examination of the first mouse, killed on day 120, showed pulmonary congestion, edema, and emphysema. The second, third, and fourth mice died on days 315, 317, and 466, respectively; they

Table 1. Relative prevalence of fungal structures in tobaccos. Relative prevalence (R.P.) is designated as none (0); slight (1+); moderate (2+); heavy (3+); pronounced (4+).

Alternaria spores		Dematiaceous mycelia	
No.	R.P.	No.	R.P.
	252 Cigarettes		
32	1+	32	1+
100	2+	100	2+
92	3+	92	3+
28	4+	28	4+
	*Fungal score**		
2.46		2.46	
	50 Cigars		
34	0		
14	1+	38	1+
1	2+	7	1+
1	3+	4	3+
		1	4+
	*Fungal score**		
0.38		1.36	
	Pipe tobaccos		
6	0	3	0
26	1+	39	1+
9	2+	9	2+
3	3+	3	3+
6	4+	6	4+
	*Fungal score**		
1.54		1.60	

* Value for fungal score was obtained by multiplying, in each case, the number of samples containing the respective fungal structure by the relative predominance of the structure indicated in column "R.P." and dividing the sum of the products by the number of samples tested.