Fig. 4. Once 10 sections are aligned, the slide is tilted, forcing the water to run down slowly. If any section tends to run with the water, it can be repositioned by pushing it with the hair while there is still water on the slide.

Although most of the time, only 1 row of 10 sections per slide was used, it was easy to obtain more, say up to 5 rows of 10 sections, hence 50 sections per slide.

This method for mounting sections and removing the excess water from the slides appears to be a very useful one, even in mounting routine. 0.5–1 µm Epon sections for radioautography, since deposits around the section, which results from the drying of water, were avoided. These deposits are usually responsible for an excessive background fog in the emulsion[3].

Resumen. Describese una tecnica para la obtención de cualquier número de cortes seriados de 0,5–1 µm de espessura, de material incluso en Epon, usandose una cuchilla de diamante. Ademas de su uso para seriación de cortes, la tecnica muestrase útil para la montage de cortes para radioautografias.

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A New Method for the Cytochemical Demonstration of *p*-Diphenol: O_2 Oxidoreductase (Laccase)

Phenol oxidases are widespread among plants and animals. In micro-organisms these enzymes are especially frequent in fungi capable of degrading lignin[1]. In this particular case, their most important purpose is the detoxification of heartwood toxins[2]. A direct participation in lignin decomposition could not be proved[3]. Melanin synthesis in fungi is also catalyzed by these enzymes[4]. The phenol oxidases are divided into *p*-diphenol:O_2 oxidoreductase (laccase; E.C. 1.10.3.2) and *o*-diphenol:O_2 oxidoreductase (tyrosinase; E.C. 1.10.3.1). However, these 2 enzymes cannot be clearly distinguished from each other because their substrate specificity is comparatively broad and overlaps. Laccase oxidizes *o*- and *p*-diphenols and *p*-phenylenediamine, but not monophenols, whereas tyrosinase catalyzes the oxidative degradation of mono- and *o*-diphenols, but not that of *p*-diphenols and *p*-phenylenediamine[5].

The procedures that have been developed for the cytochemical demonstration of phenol oxidases are comparatively nonspecific and do not permit a separate demonstration of laccase and tyrosinase. In this case the incubation medium consists of a buffered solution of L-dioxyphenyl-alanine (DOPA)[6]. The essential disadvantages of this technique are a nonenzymatic oxidation of the substrate and the water solubility of different intermediates[7]. In addition, L-tyrosine has been used as substrate[8].

In the course of investigations of the cytochemical detection of enzymes in fungi[9], an attempt was made to detect phenol oxidase activity with the procedure of LAIDLAW and BLACKBERG[10]. Even after prolonged incubation, no staining could be observed. Therefore, a better technique for the intracellular demonstration of phenol oxidases in fungal cells had to be developed.

SCHENK et al.[11] described a method for the stabilization and subsequent detection of the quinones that resulted from the oxidation of the oxicoumarins unbelliferone and aesculetin. The quinones are coupled with BESTHORN's[12] hydrazone(3-methyl-benzthiazolon(2)-hydrazon-hydrochloride) to an azo dye[13]. In food chemistry this procedure is used for the evaluation of phenol oxidase activity[14]. The resulting azo dye is not water-soluble and could therefore be of a certain importance for the development of a cytochemical procedure.

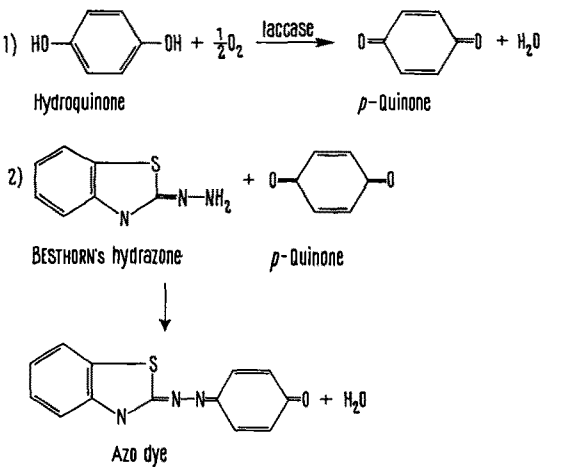
The experiments were performed with the fungi Aspergillus fumigatus (laccase producing according to[15]), Aureobasidium pullulans (laccase producing according to[16]) and Neurospora sitophila (tyrosinase producing according to[17]). Cultures on cover glasses in malt extract solution (1.7% malt extract, 0.3% peptone; Oxoid) were

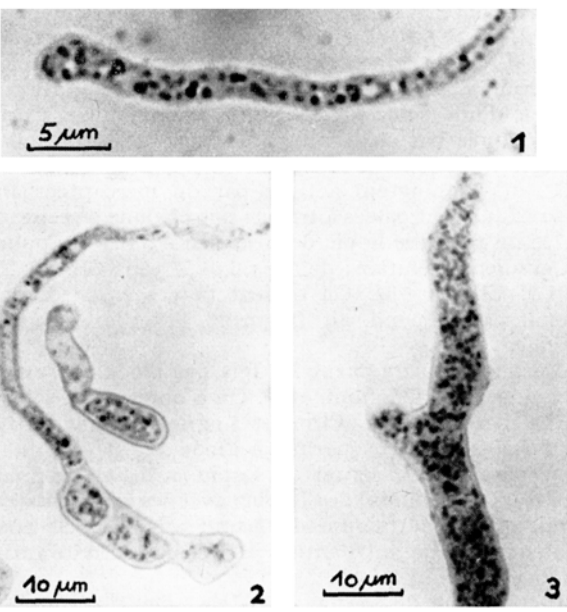
prepared according to[9]. The incubation medium contained: 0.1 ml 1% BESTHORN's hydrazone (C. Roth, Karlsruhe, Germany) in 6% ethanol, 6 ml 0.2*M* phosphate buffer (pH 7.0) and 1 ml 1% aqueous substrate solution. The substrates were hydroquinone (reinst; Merck, Darmstadt, Germany) for laccase[18] and L-tyrosine (Serva, Heidelberg, Germany) for tyrosinase[17,19]. The final pH value was 7.0 and corresponded approximately to the pH optima of fungal phenol oxidases (laccase: 6.1[19] – tyrosinase: 6–7[17,19]). The solutions of BESTHORN's hydrazone and of hydroquinone gradually change colour and must be prepared daily. The staining was performed at 37 °C in moist chambers or in staining troughs for cover glasses[20]. Controls: complete medium a) minus BESTHORN's hydrazone, b) minus substrate, c) plus $10^{-2}M$ aqueous sodium diethyldithiocarbaminate (p.a.; Merck)[5,21], d) plus $10^{-2}M$ aqueous sodium azide[5].

Results. a) *Laccase.* After the optimal incubation period of 60 min, the hyphae of all 3 fungi contained round-shaped, distinct, redbrown granules in colourless or light brown cytoplasm (Figures 1–3). The reaction products were concentrated in the growing hyphal tips; their number decreased in older parts of the mycelium with increasing age and consequent increasing vacuolation. Most of the spores of *A. fumigatus* and *N. sitophila* were stained with the same intensity; occasionally they remained unstained. No staining occurred in the controls a), b), and c), but only a weakening of the reaction picture in *A. fumigatus* and *Aur. pullulans* in control d). In *N. sitophila* the reaction was not influenced by $10^{-2}M$ sodium azide.

b) *Tyrosinase.* Even after prolonged incubation, the spores and hyphae of all 3 fungi remained unstained.

The results of the control reactions show a specificity of the tested incubation medium for the detection of *p*-diphenol:O_2 oxidoreductase (laccase). Details of the reaction mechanism resulting in the enzymatic formation of the azo dye have not as yet been described. According to HÜNIG et al.[13], the following scheme is suggested:





Cytochemical localization of laccase in *Aspergillus fumigatus* (Figure 1), *Aureobasidium pullulans* (Figure 2) and *Neurospora sitophila* (Figure 3).

[1] K. LAW, Ann. Bot. *14*, 69 (1950). – G. FÅHRAEUS, Physiologia Pl. *5*, 284 (1952). – G. LINDEBERG and G. HOLM, Physiologia Pl. *5*, 100 (1952). – T. HIGUCHI and K. KITAMURA, J. Jap. Forestry Soc. *35*, 350 (1953). – G. FÅHRAEUS, Physiologia Pl. *7*, 704 (1954). – G. LINDEBERG, Z. Pfl.-Ernähr. Düng. Bodenk. *69*, 142 (1955). – R. RÖSCH, Arch. Mikrobiol. *38*, 73 (1961); *44*, 344 (1963); *54*, 80 (1966). – G. KREBS, Arch. Mikrobiol. *40*, 94 (1961). – K. ESSER, Arch. Mikrobiol. *46*, 217 (1963). – K. ESSER, S. DICK and W. GIELEN, Arch. Mikrobiol. *48*, 306 (1964). – A. LEONOWICZ and J. TROJANOWSKI, Acta microbiol. pol. *14*, 55 (1965). – L. SCHÁNĚL, Biologia Pl. *8*, 292 (1966). – L. SCHÁNĚL, Experientia *22*, 517 (1966). – R. RÖSCH, W. LIESE and H. BERNDT, Arch. Mikrobiol. *67*, 28 (1969).

[2] H. LYR, Flora *152*, 570 (1962).

[3] R. RÖSCH, *Holz und Organismen* (Int. Symp. Berlin-Dahlem 1965), p. 173.

[4] E. C. CANTINO and E. A. HORENSTEIN, Physiologia Pl. *8*, 189 (1955). – H. M. HIRSCH, Physiologia Pl. *7*, 72 (1954).

[5] M. DIXON and E. C. WEBB, *Enzymes* (Longmans, Green and Co., London 1964). – O. H. HOFFMANN-OSTENHOF, *Enzymologie* (Springer Verlag, Wien 1954).

[6] B. BLOCH, Am. J. med. Sci. *177*, 609 (1929). – G. F. LAIDLAW, Anat. Rec. *53*, 399 (1932). – G. F. LAIDLAW and S. N. BLACKBERG, Am. J. Path. *8*, 491 (1932). – S. W. BECKER, L. L. PRAVER and H. THATCHER, Arch. Derm. Syphil. *31*, 190 (1935). – P. VAN DUIJN, J. Histochem. Cytochem. *1*, 143 (1953). – S. IIJIMA and K. WATANABE, J. invest. Dermat. *26*, 235 (1956).

[7] P. VAN DUIJN, Acta physiol. pharmac. neerl. *5*, 413 (1957).

[8] Y. YOSHIDA and Y. TOGASHI, J. invest. Dermat. *25*, 363 (1955). – C. D. MARSDEN, Q. J. microsc. Sci. *102*, 407 (1961).

[9] J. REISS, Arch. Mikrobiol. *57*, 285, 307 (1967). – J. REISS, Experientia *24*, 854 (1968). – J. REISS, Histochemie *18*, 12 (1969).

[10] G. F. LAIDLAW and S. N. BLACKBERG, Am. J. Path. *8*, 491 (1932).

[11] G. SCHENK, K.-H. FRÖMMING and H. KASTNER, Arch. Pharm. *293*, 251 (1960).

[12] E. BESTHORN, Ber. dt. chem. Ges. *43*, 1519 (1910).

[13] S. HÜNIG, H. BALLI, K. H. FRITSCH, H. HERRMANN, G. KÖBRICH, H. WERNER, E. GRIGAT, F. MÜLLER, H. NÖTHER and K.-H. OETTE, Angew. Chem. *70*, 215 (1958). – S. HÜNIG, J. chem. Educ. *46*, 734 (1969).

[14] J. VOIGT and R. NOSKE, Z. Lebensmittelunters.-Forsch. *129*, 359 (1966).

[15] E. KÜSTER and B. T. LITTLE, Biochim. biophys. Acta *67*, 288 (1963).

[16] R. RÖSCH, W. LIESE and H. BERNDT, Arch. Mikrobiol. *67*, 28 (1969).

[17] N. H. HOROWITZ and S. C. SHEN, J. biol. Chem. *197*, 513 (1952).

[18] G. FÅHRAEUS and H. LJUNGGREN, Biochim. biophys. Acta *46*, 22 (1961). – G. FÅHRAEUS, Biochim. biophys. Acta *54*, 190 (1961).

[19] J. BOIDIN, Rev. mycol. *16*, 173 (1951).

[20] J. REISS, Mikrokosmos *58*, 185 (1969).

[21] A. LEONOWICZ and J. TROJANOWSKI, Acta microbiol. pol. *14*, 55 (1965).

The azo dye is not soluble in water but in lipids, so that it is probable that it migrates into the fat vacuoles of the fungal cell after its enzymatic formation. As the site of deposition need not to be identical with the site of formation, it is impossible to assign exactly the reaction granules to individual cell particles.

The reason for the absence of any staining after incubation with L-tyrosine as substrate is very probably that all 3 fungi do not produce tyrosinase under the given conditions. This can be explained by the composition of the culture medium. Malt extract solution contains all nutrients for the most commonly occurring fungi and thus for the organisms examined in this case. HOROWITZ and SHEN[17] found that *Neurospora* shows strong tyrosinase activity only when it is grown on a sulfur-deficient medium. The tyrosinase inhibitor suspected by these authors might also to a large extent restrict enzyme production in the malt extract solution. Whether, and under what conditions, *A. fumigatus* and *Aur. pullulans* can produce tyrosinase is not known.

Zusammenfassung. Nachweis des Enzyms *p*-Diphenol: O_2 oxidoreductase (Laccase) in den Zellen der Pilze *Aspergillus fumigatus, Aureobasidium pullulans* und *Neurospora sitophila* durch einen Azofarbstoff, der mittels Kupplung des enzymatisch gebildeten *p*-Chinons mit BESTHORN's Hydrazon(3-Methyl-benzthiazolon(2)-hydrazon-hydrochlorid) entsteht. Als Substrat wird Hydrochinon verwendet. Der Farbstoff wird in runden, rotbraunen Granula abgelagert. Kontrollreaktionen bestätigen die Spezifität der Reaktion.

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Microméthode de dosage des anticorps antiallotype chez la Souris par un test de liaison avec le fragment Fc marqué à l'iode radioactif

Il s'agit de doser dans un sérum de Souris C 57 Bl/6 les anticorps anti-Igla, c'est-à-dire anti-IgG de Souris CBA. Le déterminant Igla se trouve sur le fragment Fc de l'IgG de Souris CBA. On peut marquer ce fragment Fc par l'iode radioactif. Les anticorps anti-Igla doivent se lier aux fragments Fc marqués de l'IgG Igla. Il est possible ensuite de précipiter les anticorps liés, sans précipiter les fragments Fc non liés, grâce à un sérum de Lapin anti Fab de Souris.

La mesure de la radioactivité du précipité permettra de déduire la capacité de liaison avec l'allotype Igla des anticorps d'un sérum anti-Igla.

La courbe de la radioactivité du précipité en fonction de la dilution d'un sérum anti-Igla de référence de titre connu permet aussi de doser l'activité d'un sérum anti-Igla en pourcentage d'activité du sérum de référence.

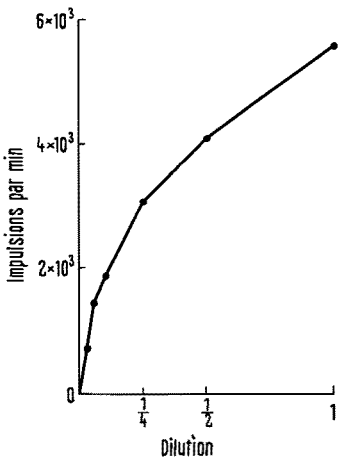
Matériel et méthodes. Préparation des fragments Fab et Fc d'IgG de Souris CBA. L'IgG est obtenue à partir de sérum normal de Souris CBA par précipitation au moyen d'une solution de sulfate d'ammonium saturée à 35% puis par chromatographie sur DEAE Cellulose. Digestion de l'IgG (100 mg) 16 h à 37 °C par la papaïne à 1% (Worthington) activée par du mercaptoéthanol. Les résidus sont passés à travers une colonne de sephadex G 75: on recueille le pic des fragments 3 S. On applique un gradient d'élution: de *Tris* 0,01 *M* sans ClNa à *Tris* 0,01 *M* ClNa 0,4 *M*. On obtient 2 pics à la sortie: le premier correspond au fragment Fab, le second au fragment Fc.

Marquage du fragment Fc Igla par l'iode 131 avec la technique à la Chloramine T[1]. On a obtenu une solution de Fc marquée à 25 µCi/ml et 5 µg/ml. Le fragment Fc peut également être marqué à l'iode 125.

Préparation du sérum de Lapin anti-Fab de Souris CBA. On a immunisé des Lapins avec les fragments Fab. On a obtenu un sérum de Lapin anti-Fab de Souris contenant 2,7 mg/ml d'anticorps avec une activité anti-Fc inférieure à 10%.

Préparation du sérum anti-Igla. On immunise des Souris C 57 Bl/6 avec des suspensions de 2×10^9 bacilles *Hémophilus pertussis* formolés agglutinés par des anticorps anti-*Hémophilus pertussis* développés chez des Souris CBA. On teste les sérums immuns C 57 Bl/6 contre du sérum CBA par précipitation en gel d'agar suivant la technique d'OUCHTERLONY[2], qui montre un trait de précipitation.

Méthode de microdosage des anticorps antiallotype. A 10 µl de solution de sérum-albumine de bœuf à 1 mg/ml contenant 8 µg/ml de fragment Fc non marqué, on mélange 10 µl de solution de Fc marqué par l'iode 131 à 5 µg/ml. Ces concentrations en fragment Fc ont été trouvées optimales. On ajoute 10 µl de sérum anti-Igla à diverses dilutions dans du sérum normal de Souris



Radioactivité, exprimée en impulsions par minute, des fragments Fc (Igla) marqués par ^{131}I précipités de façon spécifique par liaison aux anticorps anti-Igla grâce au sérum anti-Fab, en fonction de la dilution d'un serum anti-Igla.

[1] F. C. GREENWOOD, W. M. HUNTER et J. S. CLOVER, Biochem. J. *89*, 114 (1963).

[2] O. OUCHTERLONY, Progr. Allergy *5*, 1 (1958).