Table 2. The plasma radioactivity in the fibrin clot (N = 3). Incubation at 37 °C

	$^{99m}TcO_4^-$	^{99m}Tc-HSA	^{125}J-HF	^{99m}Tc-HF Complex A	^{99m}Tc-HF Complex B
3 min	0.1 ± 0.1%	0.9 ± 0.5%	89.1 ± 3.0%	74.3 ± 4.6%	66.0 ± 8.9%
10 min	0.6 ± 0.9%	1.8 ± 0.9%	88.9 ± 3.0%	39.3 ± 2.6%	65.6 ± 8.8%
3 h	0.4 ± 0.5%	1.7 ± 0.6%	86.4 ± 2.5%	2.7 ± 1.2%	55.3 ± 8.0%

Table 3. The dissociation of ^{99m}Tc-HF in human plasma at 37 °C. K (dissociation constant in sec^{-1}), A (amplitude), V (variation coefficient of the regression line), N (number of curves).

	K	A	V	N
Complex A	$1.13 \pm 0.11\ 10^{-3}$	0.855 ± 0.073	3.4 ± 0.4%	3
	$1.18 \pm 0.24\ 10^{-4}$	0.092 ± 0.009	7.2 ± 4.9%	3
Complex B	$1.07 \pm 0.23\ 10^{-5}$	0.654 ± 0.107	2.3 ± 1.3%	7

tated (table 2). The difference 3 h after incubation at 37 °C was small. Thus we could neglect fibrinolytic or other decomposing effects. A second wash of the clot with 2 ml saline solution removed only about 1.5% radioactivity, compared with the plasma sample, and could therefore be omitted.

The behavior of the two ^{99m}Tc-HF preparations in human plasma differed considerably (table 2). Complex A lost its Tc much more rapidly than complex B. It was possible to express the dissociation rate by a simple exponential function. While the complex A showed 2 phases in the first 3 h with half-lives of 10.2 min and 1.6 h (fig.), the complex B showed 1 phase with a half-life of 17.9 h. The dissociation constants and amplitudes are listed in table 3. The amplitudes mean that 85.5 ± 7.3% resp. 65.4 ± 10.7% radioactivity was bound to HF when the complex A or B had been added to the plasma (t = 0). While the amplitude for complex A was in good agreement with the results of column chromatography mentioned above, the amplitude for complex B was substantially lower, but confirming the reported isotopic clottability. Either the chromatographic separation was not effective enough in the latter case or a considerable part of the activity was bound to the fibrinopeptides which are split off at coagulation.

It was suspected that citrate could compete with proteins for Tc, which would explain the low labeling-yields found in the presence of citrate[5]. Our results with the complex A, however, show that there is no doubt about the labeling of HF with Tc even in acceptable yields (> 80%), but rapid dissociation takes place in plasma. The instability of complex A and the low labeling yield of complex B do not recommend these products for in vivo use.

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Formation of 2-methoxy-1,4-benzoquinone from vanillic acid by fungal laccases at various pH values[1]

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Summary. Laccases of various fungi metabolize vanillic acid, but contradictory reports were found concerning the formation of 2-methoxy-1,4-benzoquinone. We could establish that the pH of the medium was the major factor affecting the formation of this product.

Several methoxyphenolic acids have been reported as degradation products of lignin by various wood-rotting fungi and other microorganisms[2]. In particular, vanillic acid and its derivatives were found to result from softwood lignin biotransformation[3]. These compounds, however, are not the end products; they can be further transformed by various enzymes such as fungal phenol oxidases[4], and peroxidases[5], which may cause the formation of humic acid-like polymers[6].

Among phenol oxidases a fungal laccase (benzenediol:oxygen oxidoreductase, E.C. 1.10.3.2) can be involved in the transformation of vanillic acid. The formation of different products was established, but there exists a controversy concerning the generation of methoxyhydroquinone or methoxybenzoquinone. Several researchers determined methoxyhydroquinone as an intermediate of vanillic acid metabolism in media of laccase-producing fungi[7,8] or as a product of a partially purified enzyme[9]. During incubation of vanillic acid with a laccase of *Trametes sanguineus* at pH 4.0, 2-methoxy-1,4-benzoquinone was produced[10], whereas the same substrate incubated with a laccase of *Rhizoctonia praticola* at pH 6.9 did not form this metabolite[4]. This report attempts to clarify whether the pH or the specificity of a particular laccase is the actual factor causing the formation of methoxybenzoquinone as a product.

Laccases were isolated from the culture media of the following fungi: *Botrytis cinerea, Fomes annosus, Pholiota mutabilis, Pleurotus ostreatus, Podospora anserina, Rhizoctonia praticola* and *Trametes versicolor*. We received the culture of *B. cinerea* from Dr B. Donèche, *F. annosus* from Dr A. Hüttermann, and *P. anserina* from Dr K. Esser. The other fungi were from our own laboratories[4,11]. All cultures were grown on the same basal medium which was developed from Czapek-Dox and Lindeberg media[12], and the laccases were isolated from the culture filtrate as described earlier[13]. Laccase activity was determined on a Bausch and Lomb Spectronic 2000 spectrophoto-

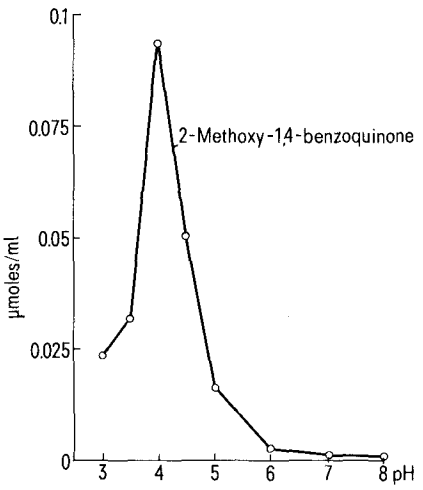

Figure 1. Formation of 2-methoxy-1,4-benzoquinone from vanillic acid by *Trametes versicolor* laccase at various pH values. The reaction mixture contained in 0.1 M citrate-phosphate buffer, 0.6 µmole/ml of vanillic acid and laccase at the concentrations described in the text. After 1 h incubation at 24 °C, the samples were acidified to pH 2 with 6 N HCl, extracted with an equal volume of methylene chloride, evaporated, dissolved in methanol, passed through a Sep-Pak C^{18} and analyzed with HPLC.

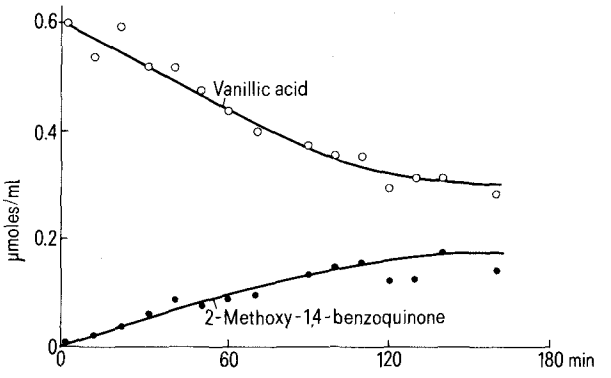

Figure 2. Pattern of vanillic acid disappearance and 2-methoxy-1,4-benzoquinone formation in an assay at pH 4.0 with the laccase of *T. versicolor*. Reaction conditions and procedures as in figure 1.

meter with 2,6-dimethoxyphenol as the substrate. One unit of the enzyme was defined as that amount which produced a change in optical density of 1.0 unit min^{-1} at 486 nm at the optimal pH of each laccase in 3.5 ml of a standard mixture containing 0.1 M citrate-phosphate buffer[14] and 3.24 µmol 2,6-dimethoxyphenol at 24 °C. Each laccase was incubated in 0.1 M citrate-phosphate buffer with 0.6 µmole/ml of vanillic acid at various pH values.

In order to produce 2-methoxy-1,4-benzoquinone for identification, 2 l of 0.1 M citrate-phosphate buffer, pH 4, containing 400 mg of vanillic acid and 2000 units of *T. versicolor* laccase were incubated for 2 h at 35 °C under vigorous agitation. The assay mixture was acidified to pH 2 with 6 N HCl and extracted with an equal volume of methylene chloride. The concentrated extract was analyzed by thin-layer chromatography on silica gel F-254 plates using a benzene:dioxane:acetic acid (90:25:4, v/v) solvent system. A yellow band of R_f 0.8 was scraped from the plate and extracted with methylene chloride. The extract was eluted through a Na_2SO_4 column and evaporated to dryness. The resulting yellow material was dissolved in methanol and eluted through a Sep-Pak C^{18} prior to high performance liquid chromatography (HPLC) analysis. The product identification of 2-methoxy-1,4-benzoquinone (MQ) was based on mass spectrometric and nuclear magnetic resonance analysis[12].

In order to establish the formation of MQ from vanillic acid at different pH values, HPLC was used as a separation technique and the yield was determined by a data module (Waters Associates, Inc., Milford, Massachusetts) by using the external standard method. HPLC was performed with a reverse phase µBondapack C-18 column with a mobile phase consisting of 20 vol of acetonitrile and 80 vol of 1% acetic acid.

Previously, the pH-dependent metabolism of vanillic acid by 2 laccases was determined[12]. For the present experiment we calculated the enzyme concentration (units/ml) for each pH value, accordingly, in order to obtain an equal disappearance of the substrate. In the case of *T. versicolor* the following amounts of enzyme were used: pH 3.0, 10.8 units/ml; pH 4.0, 8.13 units/ml; pH 5.0, 0.5 units/ml; pH 6.0, 0.98 units/ml; pH 7.0, 3.84 units/ml; and pH 8.0, 11.0 units/ml of 0.1 M citrate-phosphate buffer. The results are shown in figure 1. The formation of MQ from vanillic acid showed maximal yield at pH 4. Above pH 5.0 very little or no MQ was observed. The laccases from the other fungi gave similar results.

The relationship between the disappearance of vanillic acid and the formation of MQ at pH 4 is given in figure 2. After about 2 h of incubation of vanillic acid with the laccase of *T. versicolor* at pH 4.0, the ratio of vanillic acid to MQ present in the incubation mixture was approximately 2:1.

Our results clearly show that laccase-catalyzed transformation of vanillic acid to 2-methoxy-1,4-benzoquinone is controlled by the pH of the medium. Various laccases were investigated and in each case it was ascertained that the pH-value of the reaction mixture was the dominant factor affecting the formation of MQ. From this observation it can be implied that various products from vanillic acid may be formed in soils where fungal laccases occur and which have different pH conditions.

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