The fungus was grown on Czapek-Dox agar in petri dishes at 24 °C. The cultures (35 plates) were extracted with ethyl acetate after 14 days. The residue obtained on evaporating the solvent was extracted twice with light petroleum b.p. 40–60 °C and then recrystallized from chloroform-methanol (2:1). Bright red needles (18 mg) were obtained. Colour reactions, UV- and visible spectra and the decomposition pattern of the mass spectrum were characteristic for an anthraquinone derivative. The pigment proved to be erythroglaucin (1,4,8-trihydroxy, 3-methyl, 6-methoxyanthraquinone), since it was completely identical with preparations of erythroglaucin isolated from *Eurotium rubrum* CBS 110.31 (st. con. *Aspergillus sejunctus* = *A. ruber*[2]) and synthesized from catenarin[3].

From the mother liquor 2 other anthraquinone derivatives were isolated after chromatographing several times on preparative silica gel G layers using different solvent systems. One compound (1 mg) turned out to be catenarin (1,4,6,8-tetrahydroxy, 3-methylanthraquinone) and showed complete agreement in all respects with catenarin obtained from *Drechslera catenaria* CBS 191.29 = *Helminthosporium catenarium*[4]. The other substance (0.5 mg) could be identified as emodin (1,6,8-trihydroxy, 3-methylanthraquinone) by direct comparison with a commercial sample (Fluka).

Erythroglaucin occurs in cultures of several *Aspergillus* spp.[2] and in the lichen *Xanthoria elegans*[5]. So far it has not been detected in a species of *Talaromyces* or *Penicillium*. Catenarin was isolated from several *Drechslera* = *Helminthosporium* spp.[4], *Eurotium amstelodami* (st. con. *Aspergillus amstelodami*)[6], and only once detected in a *Penicillium* species viz. *P. islandicum*[7]. The simultaneous presence of catenarin and its 6-methylether erythroglaucin in a fungus has not been reported previously. Emodin is an ubiquitous natural anthraquinone. It occurs in both higher and lower fungi. The isolation from *Hamigera avellanea* = *Talaromyces avellaneus* (con. st. *Penicillium avellaneam*) has been described[8].

Physico-chemical data of the *T. stipitatus* metabolites[9]. Erythroglaucin: m.p. 204–206°; mol. wt. 300.06227, calc. for $C_{16}H_{12}O_6$ 300.06338[10]; λ_{max} (MeOH): 232, 256, 277, 305, 465 sh, 480 sh, 490, 510 sh, 523 nm; λ_{max} (MeOH/KOH): 245, 312, 550, 590 sh nm; ν_{max} (KBr): 1598 cm^{-1}. Catenarin: mol. wt. 286.046126, calc. for $C_{15}H_{10}O_6$ 286.047731; λ_{max} (EtOH); 232, 257, 272 sh, 282, 302 sh, 468 sh, 482, 491, 512, 525 nm; λ_{max} (EtOH/KOH): 221, 257, 300, 324, 530 nm; ν_{max} (KBr): 1600 cm^{-1}. Emodin: mol. wt. 270.053787, calc. for $C_{15}H_{10}O_5$ 270.052817; λ_{max} (MeOH): 223, 253, 267, 288, 304 sh, 438 nm; λ_{max} (MeOH/KOH): 221, 255, 310, 498 nm.

Zusammenfassung. Drei Anthrachinonpigmente wurden aus dem Schimmelpilz *Talaromyces stipitatus* CBS 349.72 isoliert. Die Untersuchung zeigte, dass sie Verbindungen identisch sind mit Erythroglaucin, Catenarin und Emodin. Durch Vergleich mit authentischen Proben konnte diese Ansicht bestätigt werden.

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Inhibition of Arylpyruvate Oxidase by Chelating Agents[1]

p-Hydroxyphenylpyruvate oxidase and phenylpyruvate oxidase have been shown to be inhibited by several chelating agents[2,3], especially diethyldithiocarbamate, which suggests that it may be a copper-containing enzyme. With some copper-containing enzymes the metal is so firmly bound that chelating agents do not remove it from the enzyme molecule[4]. Most metalloenzymes, however, are inhibited by chelating agents through the removal of the cation from the holoenzyme. The present study was undertaken to decide which mechanism applies to *p*-hydroxyphenylpyruvate oxidase.

Methods. *p*-Hydroxyphenylpyruvate oxidase[5] and phenylpyruvate oxidase[6] were assayed by the change in optical density of the enol-borate complex of the substrate. Enzyme was purified as outlined previously[7]; tests on inhibition were carried out on material purified to step (c). All reagents were the best commercially available, and distilled water was deionised to a concentration of less than 1 part per million. Copper, nickel and cobalt were assayed as complexes with diethyldithiocarbamate[8] and iron as its complex with 1,10-phenanthroline[9]. Cadmium was assayed as its complex with dithizone[10]. Unless otherwise stated all buffers were 1 mM in ascorbate.

Results and discussion. The degree of inhibition of both activities by chelating agents is shown in Table I. Diethyldithiocarbamate was the most effective chelating agent tested. In each case phenylpyruvate oxidase was inhibited more completely than *p*-hydroxyphenylpyruvate.

Reactivation was observed on addition of several cations to *p*-hydroxyphenylpyruvate oxidase that had

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Table I. Enzyme inhibition by chelating agents

Compound	Concentration (μM)	Phenylpyruvate oxidase inhibition (%)	*p*-Hydroxyphenylpyruvate oxidase inhibition (%)
Diethyldithiocarbamate	7.5	98	93
	3.0	—	58
	1.5	76	—
Ethylxanthate	10.0	95	74
	4.2	78	—
1,10-Phenanthroline	34.0	91	54
	1.9	48	—
α,α-Dipyridyl	230.0	—	35
	108.0	77	—
Phenylthiourea	220.0	—	54
	44.0	69	—
Salicylaldoxime	190.0	—	63
	48.0	72	—
8-Hydroxyquinoline	230.0	95	49
	6.9	51	—

In the studies on phenylpyruvate oxidase inhibitor and enzyme were incubated for 2–5 min at pH 7 in 0.3–0.5 ml prior to addition to the assay solution. For studies on *p*-hydroxyphenylpyruvate oxidase the inhibitor was added directly. The concentration of inhibitor is that during the assay. The enzyme activity was compared with that observed when no inhibitor was used.

been inhibited with chelating agents. Sufficient cation was used to chelate with all the inhibitor if chelation is quantitative, but not enough to cause denaturation, which was confirmed in separate experiments. With diethyldithiocarbamate as inhibitor, Cu^{2+}, Ag^{+}, Hg^{2+}, Cd^{2+}, Co^{2+}, and Ni^{2+} caused reactivation. With ethylxanthate, only Cu^{2+}, Ag^{+}, and Hg^{2+} caused reactivation. After treatment with 1,10-phenanthroline Fe^{2+}, Co^{2+}, Cu^{2+} and Ni^{2+} reactivated the enzyme (Table II). Activity was not restored by Mn^{2+}, Al^{3+}, Be^{2+}, Ba^{2+}, Cr^{3+}, Ca^{2+}, Bi^{3+}, Mg^{2+}, Pb^{2+}, Fe^{3+}, Th^{4+}, Sr^{2+}, Zn^{2+}, UO_2^{2+} or MoO_4^{2-}. Reactivation was not an artifact caused by cationic destruction of substrate.

Phenylpyruvate oxidase pretreated with chelating agents was also reactivated by cations. After treatment with diethyldithiocarbamate Ni^{2+}, Cu^{2+}, Cd^{2+}, Ag^{+} and Hg^{2+} reactivated it; after ethylxanthate Ni^{2+}, and Ag^{+}; after 1,10-phenanthroline Ni^{2+}, Co^{2+} and Fe^{2+}; after α,α-dipyridyl Co^{2+}, Zn^{2+} and Fe^{2+}; after 8-hydroxyquinoline Ni^{2+}, Co^{2+}, Cd^{2+}, Hg^{2+}, Fe^{3+} and Zn^{2+}; and after phenylthiourea Ni^{2+}, Hg^{2+} and Fe^{2+} caused reactivation. The less effective chelating agents responded less to an excess of cation than the more effective ones.

After inhibition by a large excess of chelating agents, a portion of enzyme was desalted on a short column of Sephadex G-50[2] to remove excess reagent and any chelate formed by removal of an ion from the enzyme molecule, and the enzyme activity was measured. With ethylxanthate, 1,10-phenanthroline and diethyldithiocarbamate, 76%, 92% and 33% of *p*-hydroxyphenylpyruvate oxidase activity respectively was recovered, whereas 30%, 55% and 5% of phenylpyruvate oxidase activity respectively was recovered. After phenylthiourea, 80% of the phenylpyruvate oxidase activity was recovered. Thus, the amount of enzyme that remained inactive was directly related to the effectiveness of the chelating

Table II. Inhibition of *p*-hydroxyphenylpyruvate oxidase activity by chelating agents; Reversal by cations

Chelating agent	10 μM Ethylxanthate	7.5 μM Diethyldithiocarbamate	34 μM 1,10-Phenanthroline
Concentration of cation (μM)	33	33	67
Cation		Activity	
None	26	7	46
Cu^{2+}	87	94	77
Cd^{2+}	26	68	55
Fe^{2+}	20	8	73
Co^{2+}	24	58	86
Ni^{2+}	25	79	87
Hg^{2+}	37[a]	81	36
Ag^{+}	93	90	45

The inhibitor was added to the assay solution in the final concentration shown, followed by addition of the enzyme. After incubation for 2 min, the cation was added. All concentrations are those during assay. The observed activity was compared with that when no inhibitor was used, and the results are expressed as a percentage of that value. [a] The initial reaction rate corresponded to an activity of 67%; the lower value later may be due to inactivation by Hg^{+2}.

Table III. Assay of enzyme for metal ions

Fraction from purification	Activity *p*-hydroxy-phenylpyruvate	Phenylpyruvate oxidase	Fe		Cu	
			Protein (μmole/g)	Enzyme (μmole/U)	Protein (μmole/g)	Enzyme (μmole/U)
Homogenate	10.5	5.5	14.2	2.71	0.35	0.70
(a)	28	35	0.98	0.029	0.32	0.0093
(b)	55	100	1.32	0.025	0.51	0.0095
(c)	158	210	0.65	0.0075	0.30	0.0043
(d)	210	350	0.91	0.0072	0.54	0.0043
(e)	465	690	1.21	0.0036	2.01	0.0059
(f)	560	800	0.60	0.0010	2.88	0.0052

The activity is measured in units/g of protein in a typical preparation, but not the preparation used for metal assays. Portions of enzyme from each step of the purification were treated as described in the text, and assayed for metals. Because of the quantities required for assay the materials assayed came from different batches of enzyme. The concentration of metals was compared with the concentration of protein and the activity of *p*-hydroxyphenylpyruvate oxidase.

agent. Reactivation during desalting suggested that the chelating agent did not remove a cation from the holoenzyme. To examine this hypothesis the enzyme eluted from Sephadex after inhibition by diethyldithiocarbamate was tested to determine whether the inactive portion could be reactivated by cations. *p*-Hydroxyphenylpyruvate oxidase activity was restored by the same ions that restored activity when this inhibitor was present in excess. Some of these ions even in excess were ineffective in reactivating enzyme that had been inhibited by other chelating agents which means that they did not act by replacing a cation in the apoenzyme. Thus it appears that the enzyme forms a firm complex with the chelating agent which dissociates incompletely during passage through Sephadex. The residual chelating agent is then removed from the enzyme molecule by the added cation, thus releasing active holoenzyme. Therefore, the metal in the active centre enzyme is firmly bound to the enzyme molecule.

The effect of dilution on inhibition was tested by mixing enzyme with an equal volume of 1.2 m*M* 1,10-phenanthroline, and different volumes of the mixture were assayed for *p*-hydroxyphenylpyruvate oxidase activity. When 0.1, 0.2, 0.3 and 0.5 ml portions were tested the degree of inhibition was 49%, 70%, 81% and 89% respectively. If 1 molecule of chelating agent complexes with 1 enzyme molecule the law of mass action predicts that if there is 70% inhibition with the 0.2 ml portion, there should be 56%, 78% and 85% inhibition with 0.1, 0.3 and 0.5 ml portions respectively, which is in reasonable agreement with the values observed.

Diethyldithiocarbamate and ethylxanthate were tested at 3.0 and 40 μ*M* concentration respectively to determine whether inhibition was competitive. Both inhibitors compete with substrate. They increased the apparent Km for phenylpyruvate by a factor of 2.8 and 4.0 respectively, and the Km for *p*-hydroxyphenylpyruvate by a factor of 15 and 36 respectively, without having any marked effect on V_{max}. They did not compete with ascorbate for phenylpyruvate oxidase. The chelating agents did not inhibit arylpyruvate keto-enol tautomerase.

The above results suggest that there is a firmly bound cation in the enzyme molecule. Enzyme preparations were therefore examined for some of the metal ions that reactivate inhibited enzyme, i.e. Cu, Cd, Ni, Fe, and Co. Prior to assay, the enzyme was treated with 0.1 volume of 2.5% EDTA at pH 5.5 saturated with 1,10-phenanthroline to remove loosely bound cations during passage through Sephadex. After passage through Sephadex it was wet ashed with sulfuric acid and the residue was assayed for metals. Cobalt, nickel and cadmium were not detected, but copper and iron were always present (Table III). After the removal of cytochromes in step (a), the iron content did not change appreciably on a weight basis, but decreased steadily on the basis of enzyme activity. After removal of caeruloplasmin in step (c), the copper concentration rose markedly on a weight basis, and slightly on the basis of enzyme activity. The amount of copper in a phosphate buffer taken through the whole procedure was not more than in the reagent blank. The maximum copper content, however, did not amount to more than 0.1 mole/mole of protein, based on a M. W. of about 40,000. Thus, if this enzyme contains copper, either it is still very impure, or else it loses copper irreversibly during purification. The earlier claim[11] that Fe^{2+} is essential for enzyme activity was probably based on an artifact[12].

Zusammenfassung. Die Hemmung der Phenylpyruvat- und *p*-Hydroxyphenylpyruvat-Oxydasen durch Chelat-Wirkstoffe wurde offenbar durch die Bildung eines Enzym-Wirkstoff-Komplexes verursacht, der bei Behandlung mit Kationen oder mit Sephadex trennbar ist. Die Reinigung des Enzyms führt zur Kupferanreicherung des Präparates, welches eine scheinbar funktionelle Einheit mit dem Enzym bildet.

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