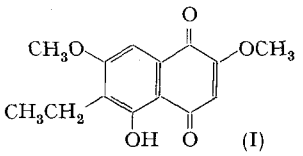
## 6-Ethyl-5-Hydroxy-2,7-Dimethoxynaphthoquinone, a Metabolite of *Hendersonula toruloidea* Nattrass

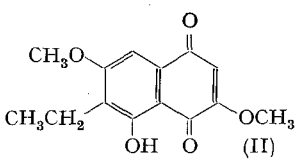
A yellow pigment, m.p. 187°, has been isolated in small yield (ca. 10 mg/l) from *Hendersonula toruloidea* Nattrass (IMI 135205). The organism was grown on a nutrient solution containing cerelose (dextrose monohydrate) (5%), ammonium tartrate (1%), $KH_2PO_4$ (0.25%), $MgSO_4.7H_2O$ (0.05%), minor elements concentrate[1] (0.1%), and yeast extract (0.1%) at natural pH. 500 ml flasks containing 200 ml sterilised nutrient solution were inoculated with *H. toruloidea* and then incubated on a rotary shaker at 25 °C for from 12–16 days. The contents of 20 flasks (3.7 l) were combined, adjusted to pH 2, and then extracted with ethyl acetate. A solution of the crude extract (2 g) in ether was washed successively with 5% aqueous sodium bicarbonate, 1 *N* sodium hydroxide, 1 *N* hydrochloric acid, and then water. The remaining ethereal solution afforded the neutral fraction (690 mg) of the initial extract. This neutral fraction was stirred with light petroleum (b.p. 40–60°) and sufficient ether to dissolve the gummy material but leave the crude pigment (53 mg) as a solid. The pigment was purified by chromatography on alumina (Grade III, neutral) using light petroleum (b.p. 60–80°) as eluting solvent, and then as crystallising solvent. Purification was monitored by TLC on silica gel GF plates. The pigment was visible as a yellow spot Rf 0.6 when the plates were developed with ethyl acetate, and Rf 0.8 with methanol-chloroform (1:9).

The mass spectrum revealed that the pigment had mol. wt. 262, and molecular formula $C_{14}H_{14}O_5$. NMR-signals ($CDCl_3$, TMS) were assigned as follows $\delta$ 12.50 (singlet, OH, 1), 7.30 (singlet, Ar-H, 1), 6.04 (singlet, $>C=C<^{H}$, 1), 4.00 (singlet, $OCH_3$, 3), 3.92 (singlet, $OCH_3$, 3), 2.75 (quartet, $J$ = 7.5 cps, $-CH_2CH_3$, 2), and 1.13 (triplet, $J$ = 7.5 cps, $-CH_2CH_3$, 3). The $C_{10}$ carbon skeleton, the colour of the compound, and the nature of the substituents suggested a naphthoquinone structure, and the evidence could be accommodated by either of structures (I) and (II)[2]. (I) has recently been obtained[3] by methylation of 6-ethyl-2,5,7-trihydroxynaphthoquinone, a product of the sodium borohydride reduction of spinachrome A. Comparison of NMR-spectra suggested that the pigment from *H. toruloidea* was in fact (I), and this was confirmed by comparison of mass spectra, by TLC, and by mixed m.p. with a sample of (I) kindly supplied by Professor P. J. Scheuer. Thus (I), although a known compound, is a new natural product. This appears to be the first isolation from a microorganism of an oxygenated naphthoquinone containing a C-ethyl substituent, a class of compound hitherto characteristic of echinoderms. 2,7-Dimethoxy-5-hydroxy-1,4-naphthoquinone (III) has recently been isolated from a strain of *Streptomyces*[4].

(I) is not active at 100 ppm in vitro against *Staphylococcus aureus* and *Salmonella dublin*, and it has no anticoagulant activity.



(I)



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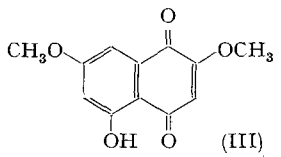

(III)

*Zusammenfassung.* Ein neuer Naturstoff, 6-Ethyl-5-hydroxy-2,7-dimethoxynaphtochinon ist aus *H. toruloidea* isoliert worden. Dieses Produkt wurde früher bei der Methylierung des 6-Ethyl-2,5,7-trihydroxynaphtochinons, welches bei der Reduktion des Spinachroms A mittels Natrium Borhydrid anfiel, erhalten.

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## Induced Peroxidase Isoenzyme Patterns in Citrus Leaves

Decreased peroxidase activity of citrus leaves was suggested as an indicator for diagnosing iron deficiency in citrus leaves[1,2]. Infiltration of iron sulphate solution into excised but intact iron-deficient citrus leaves caused part or full restoration of the enzyme activity in these leaves. This iron-restored or induced enzyme activity was negatively associated with the iron level of the substrate and the growth response of the trees, and it was therefore proposed as a measure for the determination of the iron requirement of citrus trees[3]. The occurrence of peroxidase in multiple molecular form was recognized long ago[4], and more recently the molecular heterogeneity of this enzymes was studied in further details[5,6]. Hence, it wa thought that a study of the physico-chemical properties of the enzyme by means of the separation of its various isoenzymes, might improve the application of the enzyme assay as a diagnostic agent and bring about a better understanding of the process taking place during the induction procedure of the enzyme.

Excised, intact citrus leaves were placed in 0.5% ferrous sulphate solution, or in water as a blank treatment, in Buchner flasks under vacuum suction with slight bubbling, for 3 min. After this, they were removed from the solution and placed in petri dishes on Saran nets laid

on wet filter paper and held for 48 h in incubation at 30 °C. At the end of the incubation period, 3 g leaf material were frozen in liquid air and then pulverized in 5 ml buffer phosphate 0.05 *M* pH 7.4 containing pure quartz sand. The slurry was filtered through a double layer of cheesecloth and centrifuged for 20 min at 18,000 *g* at 20 °C. The extract was diluted in 25% sucrose solution to a protein content of 300 µg/0.1 ml and was subjected to electrophoresis in polyacrylamide gel as described by DAVIS[7] for the anionic system, and by REISFELD et al.[8] for the cationic system.

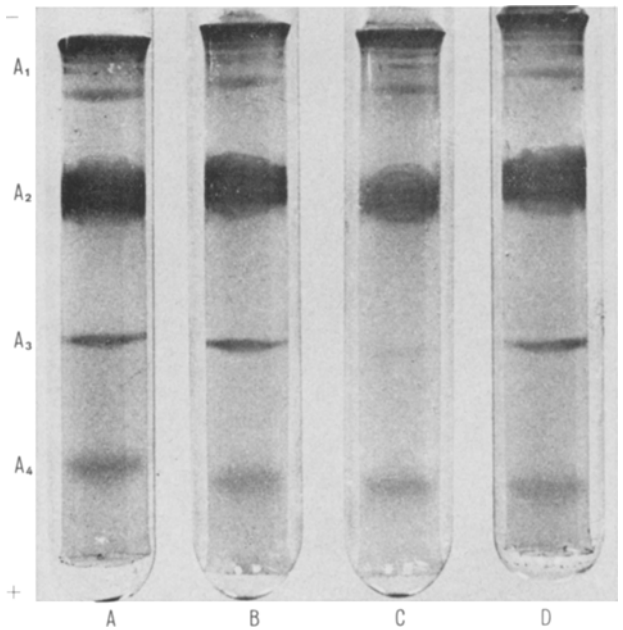


Fig. 1. Anionic peroxidase isoenzymes of lemon leaves. A, control (full nutrient) water infiltrated; B, control ferrous sulphate solution 0.5% infiltrated; C, iron deficient water infiltrated; D, iron deficient, ferrous sulphate solution 0.5% infiltrated.

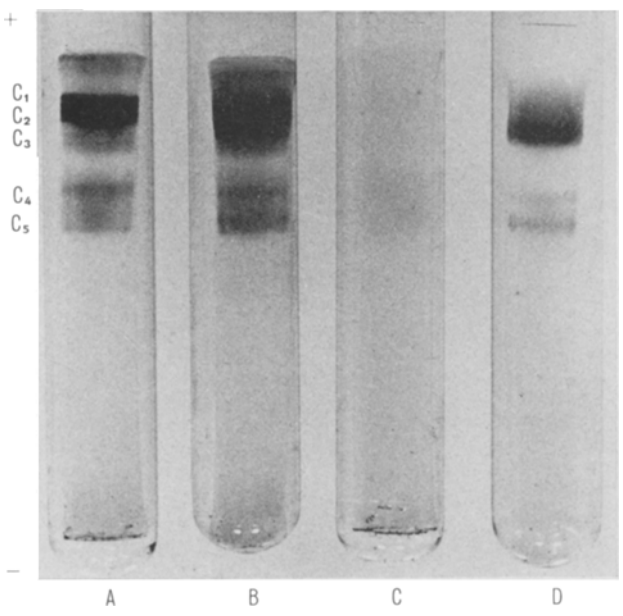


Fig. 2. Cationic peroxidase isoenzymes of lemon leaves. A, control (full nutrient) water infiltrated; B, control ferrous sulphate solution 0.5% infiltrated; C, iron deficient water infiltrated; D, iron deficient, ferrous sulphate solution 0.5% infiltrated.

Peroxidase activity was detected on the gel surface by a mixture of benzidine-guaicol and $H_2O_2$ solution[9]. The peroxidase activity, which produced coloured bands, was photographed for permanent records.

It can be seen that 4 isoenzymes $A_1$, $A_2$, $A_3$, $A_4$ moved towards the anode (Figure 1) and 5 towards the cathode (Figure 2). The quantitative differences which apparently exist between the control (full nutrient) and iron-deficient leaf extract were not measured, and we refer only to the more outstanding quantitative differences. In this regard it is interesting to note that the loss of peroxidase activity due to iron deficiency is not uniform among the different isoenzymes. The diminution of cationic peroxidase is almost total and, on the contrary, some of the anionic isoenzymes were apparently unaffected or affected only slightly. However, one of the anionic isoenzymes, $A_3$, was completely missing in the iron-deficient leaves. It appears also that the induction of peroxidase activity by means of iron, restores almost the full activity of anionic isoenzymes whilst only partly that of the cationic isoenzymes. The differences indicate that the isoenzyme might be of importance for diagnostic purposes of nutrition disorders. From a theoretical point of view it is rather unlikely that the different patterns stem from differences in metal requirements, since it has been shown that all the peroxidase isoenzymes, at least in the case of the horseradish plant, contain protohemin as the prosthetic group[6], and apparently they are not differentiated in substrate specificity[5].

At any rate, the induced peroxidase isoenzymes by means of iron infiltration into iron-deficient leaves may help in clarifying the question as to whether the enzyme induction involves de novo synthesis of the missing isoenzymes or is merely the conversion of some preformed molecule or molecules of protein and porphyrin groups into an active enzyme by means of the addition of iron to the prosthetic group[10,11].

*Résumé.* Dans des extraits de feuilles de citronnier normales et pauvres en fer, les isoenzymes de peroxidase isolés au moyen du gel d'électrophorèse, ont montré des différences quantitatives et qualitatives. L'infiltration de solution de sel de fer dans des feuilles détachées, mais intactes, a rétabli l'état presque normal en ce qui concerne les isoenzymes.

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[11] Contribution from The Volcani Institute of Agricultural Research (N.U.I.A.), Bet Dagan, Israel. 1968 Series, No. 1412-E.