ACTA CHEMICA SCANDINAVICA 22 (1968) 2557–2564

Oxidation of Methoxy-*p*-benzohydroquinone by a Lignanolytic *Agrobacterium* Strain

VERONICA SUNDMAN, HARRIET SEDERHOLM and KATRI HARO

Department of Microbiology, University of Helsinki, Helsinki 71, Finland

Methoxy-*p*-benzoquinone is a metabolite of bacterial cyclolignan degradation. In order to elucidate the biochemical pathway conducting to this metabolite the bacterial activity on methoxy-*p*-benzohydroquinone was investigated. It was demonstrated in respirometric experiments with intact washed organisms of a cyclolignanolytic *Agrobacterium* strain that methoxy-*p*-benzohydroquinone serves as hydrogen donor for the oxidative activity of the organisms, in spite of the fact that some 70 % of the respiration of the bacteria — calculated as output of carbon dioxide — is inhibited by this compound. The concentration limit of inhibition of respiration was evaluated as 0.5×10^{-3} M from the respirometric data. Smaller concentrations of methoxy-*p*-benzohydroquinone stimulated the respiration of the agrobacteria.

The total oxygen uptake corresponds to one atom of oxygen per molecule of methoxy-*p*-benzohydroquinone. However, the corresponding quinone, methoxy-*p*-benzoquinone, is not the result of this oxidation. Hence it was concluded that methoxy-*p*-benzohydroquinone does not precede methoxy-*p*-benzoquinone in the metabolic pathway of cyclolignan oxidation by the bacteria investigated.

It is known that methoxy-*p*-benzoquinone (MeQ) is an intermediate in the oxidative bacterial and fungal decomposition of guaiacyl compounds related to lignin. Sundman and Haro [1] demonstrated that during the decomposition of the cyclolignan α-conidendrin by agrobacteria, MeQ appears as a metabolite. Traces of methoxy-*p*-benzohydroquinone (MeHQ) could also be detected. Work by Fukuzumi *et al.*[2] showed that enzymes of wood-rotting fungi oxidize guaiacyl compounds to MeQ, but that MeHQ is not an intermediate in this oxidation. In order to investigate whether the same holds true for the liganolytic agrobacteria, or whether their oxidation of α-conidendrin to MeQ takes place by way of MeHQ, some experiments were undertaken to elucidate the bacterial oxidation of MeHQ.

MATERIALS AND METHODS

Bacteria. The *Agrobacterium* strain K 17, which has previously been described (Sundman [3]) as actively lignanolytic, was used throughout the work. *Escherichia coli* 113—3, a B_{12} vitamin-requiring strain, and *Bacillus megaterium* A Im6 were from the culture collection of this institution. The bacteria were grown on KYE agar in Roux bottles as previously described (Sundman and Haro [1]), washed three times with M/15 phosphate buffer pH 5.9, resuspended in the same buffer to give 10 mg dry weight of bacteria per ml, and stored in the refrigerator until used in the experiments.

Manometry. The manometric measurements were run in duplicate at 28° in air, using 15 ml flasks filled with 1 ml of a freshly prepared aqueous solution containing from 0.1 to 15 μmoles of the compound to be tested, 0.5 ml of the abovementioned bacterial suspension to be tipped from the side-arm, and 0.2 ml of 20 % potassium hydroxide solution in the centre well. The carbon dioxide produced was calculated according to the "direct" method of Umbreit *et al.*[4]

Thin layer chromatography. Fluorescent silica gel HF_{254}, manufactured by Merck AG., Darmstadt, was mixed with M/15 phosphate buffer, pH 5.0, and used as 0.25 or 0.50 mm layers. The chromatograms were developed with chloroform:benzene-ethanol, 120:40:25.

Compounds used. MeQ and MeHQ were synthesized in the way previously described by Sundman and Haro.[1] Catechol, *p*-benzohydroquinone, *p*-phenylenediamine, and guaiacol were commercial preparations of *puriss.* grade. The laccase preparation of fungal origin was isolated by Dr. G. Fåhraeus in Uppsala, Sweden, from *Polyporus versicolor* according to the method developed by Fåhraeus *et al.*[5] The horse-radish peroxidase was a commercial preparation from Boehringer & Soehne, Mannheim, Western Germany.

RESULTS

Triphasic effect of MeHQ concentration on oxygen uptake. Small amounts (0.1—0.5 μmoles) of MeHQ corresponding to the final concentrations 6.7×10^{-5} —3.3×10^{-4} M stimulated the respiration of washed *Agrobacterium* organisms. Increased amounts (1—2.5 μmoles) depressed respiration, and still larger amounts (5—15 μmoles) caused increased oxygen uptake, the net amount of which approached half a molecule of oxygen for each molecule of MeHQ (Fig. 1). This triphasic effect of the concentration of MeHQ on oxygen uptake implies that at concentrations which far exceed the concentration limit for inhibition of respiration, oxidation of the inhibiting compound occurs. The stimulation of respiration by MeHQ at lower concentrations is in accordance with the known stimulation of bacterial growth by various quinones and hydroquinones (see Webb [6]).

Inhibition of endogenous respiration with Agrobacterium *by MeHQ as measured with the aid of CO_2 output.* The curves of net oxygen uptake shown in Fig. 1 give no information on how the respiration of the washed bacteria is influenced by various amounts of MeHQ. Neither do figures for total oxygen uptake give such information, since the oxygen consumed for oxidation of MeHQ interferes with the depression of respiratory oxygen uptake. If, however, CO_2 output is registered instead of O_2 uptake, the influence of MeHQ on respiration can be visualized. The respiratory quotient of the washed bacteria approaches 1, whereas with increasing amounts of MeHQ the quotient approaches zero, as shown in Fig. 2. This implies that the oxidation of MeHQ by the bacteria is a reaction in which no CO_2 is given off. Therefore respiration in the presence of MeHQ can be followed without interference with MeHQ

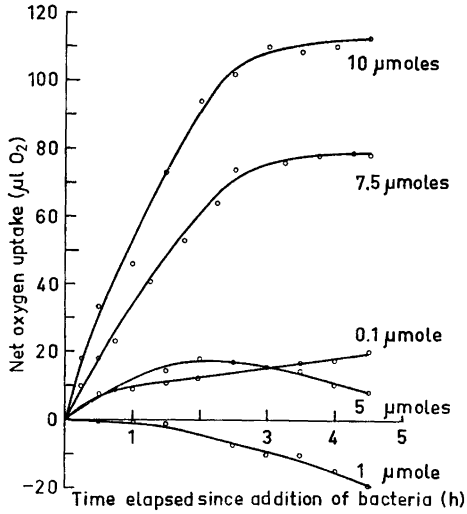

Fig. 1. Oxygen uptake with increasing amounts of MeHQ by washed intact *Agrobacterium* organisms (corrected for endogenous respiration and autoxidation of MeHQ).

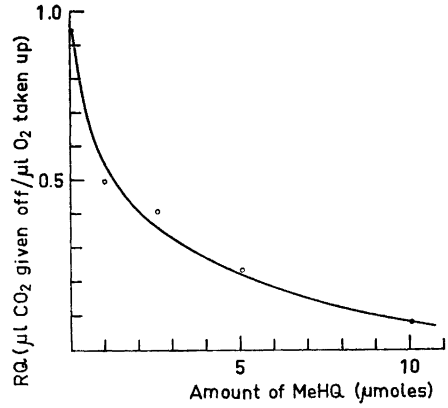

Fig. 2. Respiratory quotient, RQ, of washed intact *Agrobacterium* organisms with increasing amounts of MeHQ.

oxidation if CO_2 output is registered. Table 1 shows that about 69 % of the respiration of the washed agrobacteria is inhibited if 1 µmole MeHQ is added to the Warburg flasks. Even a tenfold increase in the amount of MeHQ does not significantly change the degree of inhibition, whereas addition of as little as 0.5 µmoles of MeHQ causes a 17 % stimulation of respiratory CO_2 output.

If a 69 % inhibition of endogenous respiration according to Table 1 was taken into account in calculations of stoichiometric oxygen uptake with MeHQ, the results obtained were those given in Table 2. It is evident that about one atom of oxygen is consumed per molecule of MeHQ, provided that more

Table 1. Influence of increasing amounts of MeHQ on the respiration of washed intact *Agrobacterium* K 17 organisms as calculated from CO_2 output in respirometric experiments during 4.5 h.

CO_2 output, µl		Amount of MeHQ		Change of CO_2 output caused by addition of MeHQ	
endogenous respiration	in the presence of MeHQ	µmoles added	molar concentration × 10^3		
58	68	0.5	0.33	+17.0 %	
58	17	1.0	0.67	−70.7 %	mean 68.9 %
68	22	2.5	1.65	−67.6 %	
68	20	5.0	3.33	−70.6 %	
36	12	10.0	6.67	−66.7 %	

Table 2. Stoichiometric oxygen uptake with increasing amount of MeHQ by washed intact *Agrobacterium* organisms. The stoichiometric oxygen uptake was calculated on the assumption that 69 % of the endogenous respiration is inhibited by the MeHQ added, using the following formula, where a = net total oxygen uptake in μl, b = corresponding endogenous uptake, c = amount of MeHQ in micromoles, and d = stoichiometric oxygen uptake:

$$(a + 0.69\ b)/11.2\ c = d$$

Micromoles of MeHQ added to the Warburg flasks (c)	Stoichiometric oxygen uptake, atoms of oxygen per molecule of MeHQ (d)	
15	1.3	
10	1.2	
7.5	1.3	
5	1.0	
2.5	0.9	
2	0.9	mean: 1.02
1.5	0.6	
1.3	0.6	
1	1.1	
0.8	1.3	
0.7	2.0	
0.6	6.9	
0.5	11.0	
0.4	15.9	
0.3	23.7	
0.2	27.5	
0.1	57.0	

than 0.7 μmoles of MeHQ is added to the Warburg flasks. For smaller amounts of MeHQ the calculation leads to nonsense, *viz.* to highly increased values of stoichiometric oxygen uptake. From this it may be assumed that the limiting amount of MeHQ for inhibition of respiration by 69 % is around 0.8 μmoles, which corresponds to a MeHQ concentration of 0.5×10^{-3} M.

Effect of MeHQ on bacteria other than Agrobacterium *K 17.* The effect of MeHQ on *Agrobacterium* K 17, as described above, can be divided into three phases: 1) stimulation of respiration at concentrations lower than 0.5×10^{-3} M, 2) inhibition of some 70 % of respiration at concentrations exceeding this value, and 3) susceptibility to oxidative enzymes, which add one atom of oxygen to each molecule of MeHQ, and the activity of which is not influenced by increasing concentrations of MeHQ up to 10^{-1} M. In order to investigate whether this triphasic effect of MeHQ on *Agrobacterium* K 17 is a general feature of the compound, and observable with other bacteria as well, some respirometric experiments were undertaken with *E. coli* and *B. megaterium*. Washed bacteria of each strain were exposed to four concentrations of MeHQ varying from 0.66×10^{-2} M to 0.33×10^{-3} M, and the oxygen uptake was registered. With *E. coli* the addition of MeHQ had no effect on oxygen uptake. No stimulation or depression of respiration or oxidation of the compound could be noted. With *B. megaterium* on the other hand, all the concentrations employed depressed respiration, but neither stimulation of endogenous oxygen uptake nor oxidation of MeHQ could be detected.

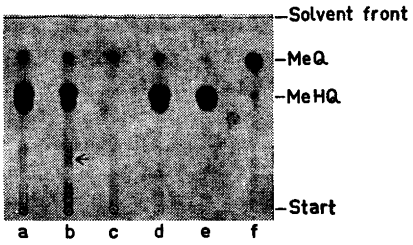

Fig. 3. Thin layer chromatograms of filtrates of shaking mixtures containing washed intact *Agrobacterium* organisms (20 mg dry weight), in 4 ml of 10^{-1} M MeHQ solution in phosphate buffer pH 5.9, shaken at 28° for various lengths of time. a. 15 min. b. 60 min, arrow indicates supposed prime oxidation product. c. 120 min. d. Sterile control, 60 min. e. Sterile control, 120 min. f. Authentic MeQ, ethanol solution applied as reference. (The unsprayed TLC plate was photographed in ultraviolet, 254 nm, illumination.)

Oxidation products of MeHQ. The oxidation of MeHQ by washed *Agrobacterium* organisms consumes about one atom of oxygen per molecule of MeHQ. Hence it is tempting to assume that the bacteria bring about the oxidative reaction MeHQ → MeQ. With the aid of thin layer chromatography, however, it was demonstrated that the disappearance of MeHQ during incubation with washed agrobacteria is not accompanied by an increase of MeQ (Fig. 3). An unstable compound (R_F 0.29) of the same yellow colour as MeQ is present in the early stages of incubation. The compound is visible in chromatograms made from filtered samples taken after 15 min and 1 h, respectively, of contact between the washed bacteria and MeHQ. After 2 h the MeHQ has disappeared almost completely, the compound at R_F 0.29 is no longer visible, and increasing amounts of a dark precipitate have formed. Slowly migrating dark compounds and water-soluble dark components which do not migrate

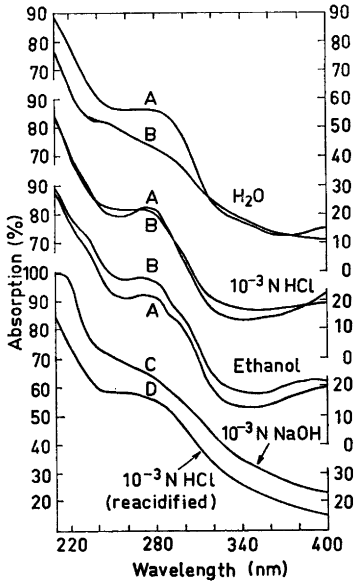

Fig. 4. Ultraviolet absorption curves in various solvents of unstable yellow metabolite of bacterial oxidation of MeHQ. *A*: Absorption scanned against solvent immediately upon elution from thin layer chromatogram. *B*: Solution was stored 24 h at 3°. *C*: Solution prepared through addition of strong hydroxide to a solution in 10^{-3} M HCl corresponding to A above. *D*: Solution C reacidified to pH 3 through addition of strong HCl.

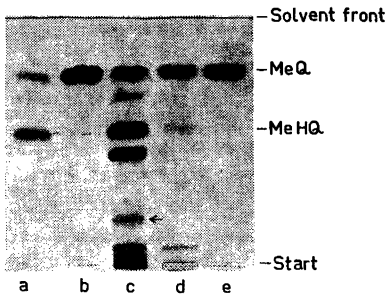

Fig. 5. Thin layer chromatograms showing oxidation products of MeHQ brought about by washed intact *Agrobacterium* organisms, horse-radish peroxidase, and laccase, respectively, at 28° in 60 min. a. Authentic MeHQ, water solution applied as reference. b. Authentic MeQ, ethanol solution applied as reference. c. Filtrate of shaking mixture of bacteria and MeHQ (equivalent to chromatogram b, Fig. 3). d. Solution of 2 μg peroxidase, 15 μl 1 % H_2O_2, and 10 μmoles of MeHQ in 1.5 ml M/15 phosphate buffer pH 5.0. e. Solution of 10 μg laccase, and 10 μmoles of MeHQ in 2.5 ml M/15 phosphate buffer pH 5.0. (Reproduction of TLC plate as in Fig. 3.)

on the chromatograms are seen around the application spots. The amount of MeQ, also present in sterile control solutions, remains unchanged (Fig. 3).

The transitory yellow compound turns brown spontaneously, like MeHQ and MeQ, if left on the chromatograms in air. It dissolves in water and ethanol but not in diethyl ether. In ethanol solution kept at —20° the compound is stable for several months, as concluded from the unchanged yellow colour of the solution and the constancy of the ultraviolet absorption 220—400 nm. Aqueous solutions are unstable, solutions in dilute hydrochloric acid somewhat more stable, but in dilute alkali the yellow colour immediately turns brown and the ultraviolet absorption maximum disappears irreversibly (Fig. 4).

MeHQ proved to serve as hydrogen donor for the enzymes laccase and horse-radish peroxidase. These oxidations were compared with the activity of the *Agrobacterium*. In manometric experiments fungal laccase in the presence of MeHQ, like agrobacteria in the presence of this compound, takes up one atom of oxygen per molecule of MeHQ. Unlike a mixture of bacteria and MeHQ, which turns blackish brown, the laccase-MeHQ solution turned bright yellow and remained so for several hours. With the aid of the thin layer chromatographic technique used, no compound other than MeQ could be demonstrated after the action of laccase on MeHQ (Fig. 5, chromatogram e). Dark polymers were not formed. Horse-radish peroxidase, on the other hand, caused a mixed oxidation. MeQ as well as slowly migrating brown compounds and non-migrating dark components were formed when peroxidase acted on MeHQ in the presence of hydrogen peroxide. The yellow compound, which is a typical product of the bacterial oxidation of MeHQ, and which is indicated by an arrow in Fig. 5, was also detected as an intermediate in the oxidation of MeHQ with peroxidase, though the compound is not visible in chromatogram d of the figure.

Specificity of the phenoloxidase activity of Agrobacterium *K 17*. The phenoloxidase activity of the *Agrobacterium* strain investigated is rather specific for MeHQ. Neither with catechol, nor *p*-benzohydroquinone, nor guaiacol could any oxygen uptake be detected in respirometric experiments in which

various amounts, from 1 to 10 μmoles, of the compounds were exposed to washed bacteria. With *p*-phenylenediamine — like the three above-mentioned compounds known as hydrogen donor with fungal laccase — the washed bacteria take up about one atom of oxygen for every molecule of the compound, and the contents of the flasks turn deep purple.

DISCUSSION

In the preceding paragraphs it was demonstrated that MeHQ serves as a hydrogen donor for phenoloxidase enzyme(s) of washed intact organisms of the lignanolytic *Agrobacterium* strain investigated, in spite of the fact that the compound inhibits some 70 % of the respiration of these bacteria. The limiting concentration of this inhibition was calculated from respirometric data to be about 0.5×10^{-3} M. This conforms well with data given by Oxford [7] on the growth-inhibiting effect of the corresponding quinone MeQ against Gram-positive bacteria. The growth of *Staphylococcus aureus* was reported to be inhibited by 0.59×10^{-3} M MeQ, whereas *E. coli* and other Gram-negative organisms were less sensitive.[6]

It has previously been stated that MeHQ is not metabolized by the agrobacteria investigated.[3] This conclusion was drawn from data on oxygen uptake in respirometric experiments in which 1—2 μmoles of MeHQ was exposed to washed bacteria. As shown in Fig. 1, the depression of respiratory oxygen uptake and the oxygen uptake due to oxidation of MeHQ balance each other with these amounts of MeHQ. Hence the net oxygen uptake will seem slightly negative or zero. The utilization of CO_2 output data instead of data on O_2 uptake, as presented in this paper, makes it possible to differentiate between respiration and phenoloxidase activity.

The reported experiments with *E. coli* and *B. megaterium* indicate that neither the phenoloxidase activity of the washed agrobacteria against MeHQ, nor their sensitivity to the respiration-depressing effect of this diphenol is a common feature among bacteria. Continued investigations will show to what extent the phenoloxidase activity demonstrated in this paper for a cyclolignanolytic *Agrobacterium* strain occurs among other soil bacteria. The oxidative polymerization of phenols, important from the point of view of humification of the soil, might be a mechanism by which organisms, including bacteria, susceptible to the growth-inhibiting effect of these compounds protect themselves from such detrimental influence (see Bortels and Henkel,[8] Lyr,[9] Mishustin *et al.*[10]).

MeQ could not, with the aid of thin layer chromatography, be demonstrated as a metabolite in the bacterial oxidation of MeHQ. It was therefore concluded that the humification which accompanies the utilization of the cyclolignan α-conidendrin by the agrobacterium investigated, and which might proceed *via* MeQ (Sundman and Haro [1]), does not involve MeHQ as an intermediate. This conforms with the mechanism proposed by Fukuzumi *et al.*[2] for the oxidation of vanillic acid to MeQ by enzymes from wood-rotting fungi, where MeHQ is not supposed to be an intermediate.

The transitory unstable yellow metabolite which appears in the culture fluid if agrobacteria are shaken with MeHQ in phosphate buffer, pH 5.9, and

the ultraviolet absorption of which is presented in Fig. 4, might possibly be a quinoid dimer of MeHQ, which readily undergoes polymerization to dark compounds.

Acknowledgements. The authors wish to express their gratitude to Dr. Gösta Fåhraeus of Uppsala, Sweden, for supplying the preparation of fungal laccase.

This work has been supported financially by grants from the National Research Council for Agriculture and Forestry (Finland), which is gratefully acknowledged.

REFERENCES

1. Sundman, V. and Haro, K. *Finska Kemistsamfundets Medd.* **75** (1966) 11.
2. Fukuzumi, T., Uranshihara, S., Oohashi, T. and Shibamoto, T. *J. Japan Wood Res. Soc.* **10** (1964) 242.
3. Sundman, V. *Acta Polytech. Scand. Chem. Met. Ser.* **40** (1965) 1.
4. Umbreit, W. W., Burris, R. H. and Stauffer, J. F. *Manometric techniques*, 2nd Ed., Burgess Publishing Co., Minneapolis 1957.
5. Fåhraeus, G., Tullander, V. and Ljunggren, H. *Physiol. Plantarum* **11** (1958) 631.
6. Webb, L. J. *Enzyme and metabolic inhibitors*, Academic, New York 1966, Vol. III, p. 543.
7. Oxford, A. E. *Chem. Ind. (London)* **61** (1942) 189.
8. Bortels, H. and Henkel, H. G. *Arch. Mikrobiol.* **60** (1968) 99.
9. Lyr, H. *Phytopathol. Z.* **52** (1965) 229.
10. Mishustin, E. N., Ezofeev, D. J., Nikitin, D. I. and Vostrov, J. S. *Ann. Inst. Pasteur. Suppl.* **3** (1964) 211.

Reveiced March 30, 1968.