Bacterial Hydroxylation of Pyrazon Compounds
E. de Frenne, J. Eberspächer, and F. Lingens
Institut für Mikrobiologie und Molekularbiologie, Universität Hohenheim, Stuttgart
and
W. Schäfer
Max-Planck-Institut für Biochemie, Martinsried

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In cell suspensions of bacteria grown with 5-amino-4-chloro-2-phenyl-3(2H)-pyridazinone (pyrazon) as sole source of carbon o-methyl-pyrazon and m-methyl-pyrazon were converted to the corresponding hydroxymethyl compounds. p-Methylpyrazon was not metabolized.

Bacteria, selected for their capacity to grow on pyrazon as sole source of carbon, have been isolated and described by Fröhner, Oltmanns, and Lingens 1. These bacteria have been shown to remove the benzene moiety of the pyrazon molecule.

This paper deals with the metabolism of pyrazon compounds, substituted on the benzene nucleus with a methyl group.

Materials and Methods

Chemicals

o-Methyl-pyrazon, m-methyl-pyrazon and p-methyl-pyrazon were supplied by BASF, Ludwigshafen, Germany.

Physical measurements

Melting points were recorded with a Büchi melting point apparatus and are uncorrected. Ultraviolet spectra were determined by the photometer DMR 21 from Carl Zeiss (Oberkochen, Germany). Infrared spectra were measured in KBr with a spectrophotograph from Ernst Leitz (Wetzlar, Germany). Mass spectra were recorded with the spectrometer CH 7 from Varian MAT (Bremen, Germany).

Abbreviations: Pyrazon, 5-amino-4-chloro-2-phenyl-3(2H)-pyridazinone; o-methyl-pyrazon, 5-amino-4-chloro-2-(2-methylphenyl)-3(2H)-pyridazinone; m-methyl-pyrazon, 5-amino-4-chloro-2-(3-methylphenyl)-3(2H)-pyridazinone; p-methyl-pyrazon, 5-amino-4-chloro-2-(4-methylphenyl)-3(2H)-pyridazinone.

Requests for reprints should be sent to Prof. Dr. F. Lingens, Institut für Mikrobiologie und Molekularbiologie der Universität, D-7000 Stuttgart-Hohenheim, Garbenstraße 30.

Bacterial growth

Strain E of the pyrazon-degrading bacteria was inoculated into a flask containing 1 l of a pyrazon-mineral salt medium. The composition of the culture medium has been described previously 1. The culture was grown on a rotary shaker for 3 d at 27 °C. This cell suspension was used as an inoculum for a laboratory fermentor containing 10 l of pyrazon-mineral salt medium. This culture was allowed to grow at 27 °C with aeration for 36 h. Cells were harvested by centrifugation and washed twice with saline. The yield was approximately 0.3 g of cells (wet weight) per l of medium.

Incubation conditions and isolation of products

4 g of cells (wet weight) were suspended in 1 l of a mineral salt medium, which had been supplemented with 300 mg of o-, m- and p-methyl-pyrazon, respectively. The suspensions were incubated for 24 h with shaking.

The bacteria were removed by centrifugation and each culture fluid was repeatedly extracted with ethylacetate. From the culture fluid, which had been supplemented with o-methyl-pyrazon compound 1, and from the culture fluid supplemented with m-methyl-pyrazon, compound 2 could be isolated. No conversion of p-methyl-pyrazon could be detected.

The extracts containing compound 1 and compound 2, respectively, were evaporated to dryness in vacuo and washed with a small amount of ether. Further purification of these compounds was achieved by gel filtration using a Sephadex LH-20 column and ethanol-water (1:1; v/v) for elution. Repeated recrystallisation of compound 1 from water yielded white crystals (m.p. 217 °C). Compound 2 after recrystallisation from water yielded long white needles (m.p. 202 °C).
Results

Compound 1, which was isolated from the culture medium supplemented with o-methyl-pyrazon, was identified as 5-amino-4-chloro-2-(2-hydroxymethyl-phenyl)-3(2H)-pyridazinone. Compound 2, isolated from the culture medium supplemented with m-methyl-pyrazon, was identified as 5-amino-4-chloro-2-(3-hydroxymethylphenyl)-3(2H)-pyridazinone.

The UV-spectra in aqueous solution showed maxima at 279 nm and 226 nm (compound 1) and at 283 nm and 227 nm (compound 2), respectively. The maxima of absorption were not affected by the addition of HCl (to pH 1) or NaOH (to pH 13), which is in agreement with the proposed formula. The UV-maxima neither of pyrazon nor of 5-amino-4-chloro-3(2H)-pyridazinone are affected by the addition of alkali or acid.

No evidence for the presence of phenolic hydroxyl groups and olefinic double bonds could be obtained using the FeCl₃ or the Folin-reagent and bromine-water, respectively. Analytical data of both substances were consistent with the formula C₁₁H₁₀N₃O₂Cl

Calcd: C, 52.50%; H, 4.00%; N, 16.70%; Cl, 14.08%.

Found for compound 1: C, 52.34%; H, 3.90%; N, 16.71%; Cl, 13.87%.

Found for compound 2: C, 52.58%; H, 3.85%; N, 16.61%; Cl, 14.13%.

The mass spectra (Fig. 1) show significant differences. The spectrum of compound 1 shows extensive fragmentation resulting from a strong ortho effect: Abundant fragments at m/e 234/236 (a), 220/222 (b) and 145/147 (e).

Compound 2 shows intense peaks at m/e 250/252 (M-1) and at m/e 107, respectively. Both fragmentation patterns are similar to that of benzylalcohol. Elimination of OH is only observed in compound 1. Elimination of CH₂OH is more pronounced in compound 1.

Discussion

From the culture medium of bacteria, that utilize 5-amino-4-chloro-2-phenyl-3(2H)-pyridazinone (pyrazon) as sole source of carbon for growth several metabolites were isolated. Using the structure of these compounds a pathway for the degradation of pyrazon could be proposed. According to this scheme the benzene nucleus of pyrazon in a first stage is attacked by an oxygenase, which catalyzes the conversion of the benzene moiety to a benzene glycol compound.

An additional substituent on the aromatic nucleus presents microorganisms with a choice as to their mode of attack. Studies on the metabolism of toluene and isopropylbenzene by *Pseudomonas putida* have shown that these compounds are converted to ortho dihydroxy compounds, in which the side chain is left intact. The identification of intermediate compounds involved in the degradation of either benzene or toluene indicated that both compounds are metabolized by *Pseudomonas putida* in the same way.

The observations presented here, demonstrate that an additional methyl substituent on the aromatic nucleus prevents the pyrazon-degrading bacteria
from an oxidative attack on the benzene moiety of the pyrazon molecule. So methylpyrazons did not support growth of these bacteria. Under the conditions of cometabolism only o- and m-methyl pyrazon are converted to the corresponding hydroxymethyl compounds, the p-isomer was not metabolized at all.

It would be of notable interest, whether the hydroxylation is catalyzed by a separate alkylhydroxylase or by the enzyme, which normally catalyzes the oxidative attack of the aromatic nucleus of pyrazon. A oxygenase which can also hydroxylate a methyl group was observed in a Pseudomonas fluorescens. An enzyme which converts benzoic acid to dihydroxybenzoic acid has been purified from this organism. This enzyme was found to transform o-methyl benzoic acid to o-hydroxymethyl benzoic acid. A similar result was obtained with a purified phenylalanine hydroxylase from Pseudomonas sp. This enzyme, which catalyzes the hydroxylation of phenylalanine to tyrosine, was found to convert p-methyl-phenylalanine in appreciable amounts to p-hydroxymethyl-phenylalanine.

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